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Cynthia Wilson

Cynthia Wilson

PATENT
Attorney's Docket No. P-061-R2

#28
15U418
1-2-03

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Burton CHRISTENSEN et al.	
	Group Art Unit: 1639
Application No.: 09/457,926	
	Examiner: M. Garcia Baker
Filed: December 8, 1999	
For: NOVEL ANTI-BACTERIAL AGENTS	

BRIEF FOR APPELLANT

Commissioner for Patents
Washington, D.C. 20231

ATTENTION: BOARD OF PATENT APPEALS AND INTERFERENCES

Sir:

This Brief is submitted in response to the final Office Action mailed on July 2, 2002, for the above-identified patent application; and in furtherance of the Notice of Appeal filed on November 4, 2002. Pursuant to 37 C.F.R. §1.192(a), this Brief is accompanied by the fee set forth in 37 C.F.R. §1.17(c) and is submitted in triplicate. Additionally, pursuant to 37 C.F.R. §1.192(c)(9), the appealed claims are set forth in APPENDIX A.

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I. REAL PARTY IN INTEREST (37 C.F.R. § 1.192(c)(1))

The real party in interest in this appeal is THERAVANCE, INC., a corporation duly organized under and pursuant to the laws of Delaware, and having its principal place of business at 901 Gateway Boulevard, South San Francisco, California 94080.

II. RELATED APPEALS AND INTERFERENCES (37 C.F.R. § 1.192(c)(2))

The Appellant, the Appellant's legal representative, or the Assignee are not aware of any other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

III. STATUS OF THE CLAIMS (37 C.F.R. § 1.192(c)(3))

Claims 41-46, 49-51, 53-55, 57 and 58 are the subject of this appeal. Of these pending claims, Claims 41, 43, 49-51 and 53-55 have been rejected and Claims 42, 44-46, 57 and 58 have been withdrawn from consideration by the Examiner but not cancelled. Claims 1-40, 47, 48, 52 and 56 have been cancelled. No claims have been allowed.

IV. STATUS OF AMENDMENTS (37 C.F.R. § 1.192(c)(4))

No amendments have been filed subsequent to the final rejection.

V. SUMMARY OF INVENTION (37 C.F.R. § 1.192(c)(5))

Appellants' claimed invention is directed to chemical compounds having the formula:

$$L'\text{-}X'\text{-}L''$$

or pharmaceutically acceptable salts thereof; wherein L′ is a specifically-defined β-lactam moiety; L″ is a vancomycin moiety (or an aglycone derivative thereof); and X′ is a specifically-defined linker as further described on page 39, line 18 to page 48, line 08 of the specification. Specific embodiments of the presently claimed invention are illustrated, for example, in Figures 9 and 10 (where the structure of the vancomycin moiety has been abbreviated as a square box as described on page 38, lines 10-17 of the specification).

In the presently claimed compounds, the β-lactam and vancomycin moieties are attached to the linker through specifically-defined points of attachment. For example, in Claim 41 (reproduced in APPENDIX A), the β-lactam moiety of formula (a) is covalently linked to the linker through substituents R, R^1 or R^2; and the vancomycin moiety (or aglycone) is linked to the linker through the carboxy terminus, the amino terminus, the dihydroxyphenyl ring, the saccharide amino group, or the aglycone hydroxy terminus of the vancomycin moiety.

The presently claimed invention is also directed to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of any of the claimed compounds as further described on page 112, line 31 to page 115, line 14 of the specification. The compounds and pharmaceutical compositions of this invention are useful as antibacterial agents as further described on page 112, lines 15-29 of the specfication.

VI. ISSUES (37 C.F.R. § 1.192(c)(6))

Whether Claims 41-46, 49-51, 53-55, 57 and 58 are unpatentable under 35 U.S.C. §103(a) over:

(1) U.S. Patent No. 5,693,791, issued on December 2, 1997 to William L. Truett ("Truett"); in view of:

(2) Michael Boeckh et al., *Antimicrob. Agents Chemother.*, **1988**, *32*(1), 92-95 ("Boeckh");

(3) M. Renoud-Grappin et al., *Antiviral Chem. and Chemotherapy*, **1998**, *9*(3), 205-223 ("Renoud-Grappin"); and

(4) Thomas Staroske and Dudley H. Williams, *Tet. Lett.* **1998**, *39*, 4917-4920 ("Staroske").

VII. GROUPING OF CLAIMS (37 C.F.R. § 1.192(c)(7))

The claims subject to appeal do not stand or fall together. Specifically, Claims 46, 54, 57 and 58 are believed to be separately and independently patentable for the reasons discussed herein below.

VIII. ARGUMENTS (37 C.F.R. § 1.192(c)(8))

The sole issue for consideration in this appeal is whether the Examiner has establish a *prima facie* case of obviousness for Appellants' claimed invention.

A. LEGAL STANDARD REQUIRED FOR ESTABLISHING A PRIMA FACIE CASE OF OBVIOUSNESS

The Examiner bears the initial burden of establishing a *prima facie* case of obviousness. *See In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). In order to satisfy this burden, the Examiner must make three showings:

(1) First, the prior art relied upon, coupled with the knowledge generally available in the art at the time of the invention, must contain some suggestion or incentive that would have motivated the skilled artisan to modify a reference or to combine references in a manner that produces the claimed invention. *See, In re Fine*, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988) (holding that a *prima facie* case of obviousness can be established only "by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings of the references."). For an invention directed to a chemical compound, the requisite motivation is generally derived from structural similarity between the prior art and the claimed invention. *See, e.g., Yamanouchi Pharm. Co., Ltd. v. Danbury Pharmacal, Inc.*, 231 F.3d 1339, 1343, 56 USPQ2d 1641, 1644 (Fed. Cir. 2000).

(2) Second, the Examiner must show that the proposed modification of the prior art references had a reasonable expectation of success as determined from the vantage point of one of ordinary skill in the art. *See Yamanouchi*, 231 F.3d at 1343, 56 USPQ2d at 1644; and *Amgen, Inc. v. Chugai Pharm. Co.*, 927 F.2d 1200, 1209, 18 USPQ2d 1016, 1023 (Fed. Cir. 1991) ("there was no reasonable expectation of success...[t]here were many pitfalls.").

Both the motivation to modify the prior art references, as well as the expectation of success, must come from the prior art, not from applicant's own disclosure. *See, In*

re Vaeck, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991).

(3) Lastly, the Examiner must show that the prior art reference or combination of references teach or suggest all the limitations of the claims. *See*, *In re Wilson*, 424 F.2d 1382, 1385, 165 USPQ 494, 496 (CCPA 1970) ("All words in a claim must be considered in judging the patentability of that claim against the prior art.").

If the Examiner fails in any of these requirements, she has not established a *prima facie* case of obviousness, and without more, the applicant is entitled to a patent. *See, e.g., Oetiker*, 977 F.2d at 1445, 24 USPQ2d at 1444 (citing *In re Grabiak*, 769 F.2d 729, 733, 226 USPQ 870, 873 (Fed. Cir. 1985)).

B. THE EXAMINER'S REJECTION

The Examiner has concluded that Appellants' claimed invention is *prima facie* obvious in view of the cited references for the following reasons:

> [I]t would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention to link vancomycin and ceftazidime, based on the teaching of Truett concerning the linking of diverse antibiotic moieties combined with the teaching of Boeckh et al to perform combination therapy using the drugs, the teaching of Renoud-Grappin concerning linking drugs to perform combination therapy and the teaching of Staroske et al concerning vancomycin dimers linked through the amino and carboxy terminus. Specifically, Truett teaches that two antibiotics, one known to attack Gram positive bacteria and another to attack Gram negative bacteria can be linked and the advantages of doing such, and Boeckh et al teach that vancomycin and ceftazidime fulfill these requirements. Renoud-Grappin teach that one way to achieve effective combination therapy is to covalently link two different drugs. Finally, Staroske et al teach that vancomycin can be linked at specific linkage sites. One of ordinary skill would have been motivated to covalently link vancomycin with ceftazidime to create a broad spectrum antibiotic compound to fight antibiotic resistant strains. One of ordinary skill would also have had a reasonable expectation of success based on the fact that Staroske et al teaches linking chemistry for vancomycin. Final Rejection, U.S. Serial No. 09/457,926, at pages 5-6 (July 2, 2002).

For the reasons discussed herein, the Examiner's arguments as summarized above

fail to establish a *prima facie* case of obviousness and therefore, the rejection of Appellants' claimed subject matter should be reversed.

C. CITED REFERENCES DO NOT PROVIDE REQUISITE MOTIVATION

In proving a *prima facie* case of obviousness, the Examiner must show that the prior art relied upon, coupled with the knowledge generally available in the art at the time of the invention, provided some suggestion or incentive that would have motivated the skilled artisan to modify the references in a manner that would have produced the claimed invention. In the present case, this requisite motivation is lacking for the following reasons.

1. STRUCTURAL SIMILARITY DOES NOT PROVIDE MOTIVATION FOR PROPOSED MODIFICATION

Appellants first note that the requisite motivation to modify the cited references is not provided by any structural similarity between the compounds disclosed in the cited references and Appellants' claimed compounds, i.e., Appellants' claimed compounds are not homologs, analogs or isomers of any of the prior art compounds of record. In *Yamanouchi*, the Federal Circuit stated that "[f]or a chemical compound, a *prima facie* case of obviousness requires 'structural similarity between claimed and prior art subject matter...where the prior art gives reason or motivation to make the claimed compositions.'" *Yamanouchi*, 231 F.3d at 1343, 56 USPQ2d at 1644 (Fed. Cir. 2000), quoting *In re Dillon*, 919 F.2d 688, 692, 16 USPQ2d 1897, 1901 (Fed. Cir. 1990) (*en banc*). In the present case, no such structural similarity exists. Accordingly, structural similarity does not provide the requisite motivation or suggestion to modify the prior art compounds in a manner which produces Appellants' claimed invention.

2. CITED REFERENCES FAIL TO PROVIDE MOTIVATION TO MAKE THE PROPOSED MODIFICATION

In the absence of structural similarity, the Examiner has attempted to piece together elements present in the prior art to construct Appellants' claimed subject matter. However, the cited references fail to provide the requisite motivation to combine such

elements in a manner that produces Appellants' claimed invention.

Specifically, the primary reference relied upon by the Examiner to establish a *prima facie* case of obviousness is the Truett reference. This reference discloses that various antibiotic moieties can be linked together using diisocyanates, dianhydrides, diacid chlorides, diepoxides and carbodiimides (*see* Truett at Column 1, lines 5-15). However, Truett teaches that:

> The types of antibiotics that can be linked are sulfonamides, trimethoprim, penicillins and related structures, cephalosporins and related structures, chloramphicol, erythromcyin, metronidiazole, quinolones, tetracyclines and aminoglycosides. Truett at Column 6, lines 34-38.

Thus, Truett does not teach or suggest the use a glycopeptide antibiotic, such as vancomycin, for the preparation of antibiotic dimers. Since this reference lacks any disclosure of a glycopeptide antibiotic, it does not provide the necessary motivation on its own to link vancomycin to a β-lactam antibiotic and thus, produce Appellants' claimed invention.

Recognizing this limitation, the Examiner has first combined the Truett reference with the Boeckh reference. Boeckh teaches the use of physical mixtures of vancomycin and ceftazidime (a β-lactam) to treat bacterial infections (*see* Boeckh at page 92, first paragraph). In such physical mixtures, the vancomycin and ceftazidime are separate chemical entities not linked together but merely mixed as two separate components. Thus, the Boeckh reference does not disclose or suggest in any way the use of antibiotic dimers. Accordingly, on its own, the Boeckh reference cannot provide the necessary motivation to chemically link vancomycin to ceftazidime.

In combining these references, however, the Examiner has argued that:

> Truett teaches that two antibiotics, one known to attack Gram positive bacteria and another to attack Gram negative bacteria can be linked and the advantages of doing such, and Boeckh et al teach that vancomycin and ceftazidime fulfill these requirements. Final Rejection at page 6.

In response, Appellants first take issue with the Examiner's statement that Truett

teaches the advantages of linking two antibiotics together. Specifically, Appellants respectfully point out that the Truett reference does not explicitly state any particular advantage to be gained by linking antibiotic compounds together. At best, Truett states that:

> This invention is concerned with the preparation of a wide variety of antibiotics of new and novel structure and antimicrobial activity...It has been realized that the linking of two antibiotic moieties functioning in different fashions, as for example inhibiting cell-wall synthesis or protein synthesis or DNA synthesis, ***can be of value***. Two antibiotic moieties can also be linked in which one is known to attack Gram positive bacteria and another to attack Gram negative bacteria, and this new entity is ***of value***. Truett at Column 1, lines 05-07 and 24-30 (emphasis added).

Beyond stating that the linked antibiotics have "value" (i.e., presumably that such dimers have utility), no particular advantage is described by Truett for such compounds. In fact, the Truett reference provides data for only one antibiotic dimer compound and that compound is reported to have the same antibacterial activity as unlinked standard antibiotics. Specifically, Truett discloses the preparation and testing of a dimer of *p*-aminobenzene sulfonamide and sulfapyridine in Column 45, beginning at line 25. In reporting the activity of this compound, Truett states that:

> Tabs of the filter paper [containing the dimer of *p*-aminobenzene sulfonamide and sulfapyridine] were applied to agar culture plates streaked with standard bacterial cultures of *S. aureus*, *E. coli* and *P. aureginosa*. Standard antibiotics, as p-aminosulfonamide and penicillins were used for comparison. ***All products showed modest inhibition zones*** in the vicinity of the filter paper tabs containing the product fractions. Truett at Column 45, lines 57-64 (emphasis added).

Thus, not only does Truett fail to state any explicit advantage to be gained by linking antibiotic moieties together, but this reference actually demonstrates that when such dimers are prepared, the products do not have any advantage over standard antibiotics, i.e., *all* products showing "modest" inhibition zones.

In contrast, Boeckh teaches that physical mixtures of vancomycin and ceftazidime, i.e., mixtures where the components are not linked together, are highly effective for

treating bacterial infections. Specifically, Boeckh teaches:

> Recent studies demonstrate an excellent clinical response to the combination of vancomycin and ceftazidime in febrile neutropenic cancer patients.... In vitro studies with this combination showed strong synergism and prevention of secondary resistance to most staphylococci.... In summary, the combination of vancomycin and ceftazidime is an effective regimen to compensate for the poor antistaphylococcal activity of ceftazidime alone.... Boeckh at page 94, first full paragraph, right column.

Accordingly, in view of the fact that physical mixtures of vancomycin and ceftazidime are reported by Boeckh to be effective for treating bacterial infections and Truett neither reports nor demonstrates any explicit advantage for the linked antibiotics disclosed therein, the combined disclosures of these references would not provide one skilled in the art with sufficient motivation to modify their teachings in a manner which produces Appellants' claimed invention.

In this regard, it is important to recognize that preparing dimers of antibiotics requires significant extra effort on the part of the skilled artisan. Additional chemical reactions must be conducted and the resulting products typically must be purified. For example, beginning in Column 25, at line 19, Truett discloses various synthetic procedures required to prepare dimers. Truett further teaches, for example in Column 44, at lines 01-04 and 25-26, that such reactions often produce mixtures of products that require separation by chromatographic techniques (*see* Truett at Column 44, lines 01-04 and 25-26).

Thus, in view of the extra effort required to prepare dimers, one skilled in the art would find insufficient motivation to make dimers of vancomycin and ceftazidime based on the teachings of Truett since this reference neither reports nor demonstrates any explicit advantage for the linked antibiotics disclosed therein. This is especially true in view of the fact that Boeckh teaches that physical mixtures of vancomycin and ceftazidime are an "effective regimen" for the treatment of bacterial infections. Based on these teachings, one skilled in the art would clearly explore physical mixtures of antibiotics rather than chemically-linked antibiotics.

The Examiner has attempted to strengthen her argument by also citing the

Renoud-Grappin reference and arguing that:

> Renoud-Grappin teach that one way to achieve effective combination therapy is to covalently link two different drugs. See page 208, first column, first full paragraph of the reference, which describes using heterodimers for combination therapy linked "through an appropriate spacer, in an attempt to combine the inhibitory capacity" of two different classes of molecules. The reference also describes that one would attempt such an approach to span two binding sites on the target. Renoud-Grappin et al also discuss combining different drugs to "prevent the emergence of drug-resistant virus strains" and set forth three main reasons for combination therapy (see page 207, 2nd column, 2nd paragraph). It is recognized that the linked compounds of Renoud-Grappin et al (see, for example, Figure 4 of the reference) are anti-virals and not antibiotics; however, it is the examiner's position that one of ordinary skill would recognize the relevance of preventing the emergence of drug-resistant strains for both classes of molecules since such was well established in the art. Final Rejection at pages 4-5.

However, the teachings of the Renoud-Grappin reference do not cure the deficiencies of Truett and Boeckh and, in fact, actually direct the skilled artisan away from the preparation of dimers for the following reasons.

First, while Renoud-Grappin attempts to achieve effective combination therapy through the use of dimers of anti-viral agents, their actual results demonstrate that the dimer approach did not work in their case. Specifically, Renoud-Grappin teach:

> In conclusion, no synergistic antiviral activity and no diminished toxicity were found....Several reasons may explain the ***failure of this heterodimer approach*** to increase the inhibitory activity against HIV.... Renoud-Grappin at page 219, second column (emphasis added).

Accordingly, rather than providing the requisite suggestion or incentive to motivate one skilled in the art to prepare dimers of antibiotics, the Renoud-Grappin reference actually teaches away from the preparation of dimers by demonstrating that the disclosed dimers did not provide any advantage. Again, this information, when combined with the knowledge that physical mixtures of vancomycin and ceftazidime were known to be an "effective regime" for treating bacterial infections, would actually lead one skilled

in the art to explore the use of *physical mixtures* of antibiotics and not to prepare dimers because no actual advantage is demonstrated for dimers.

Additionally, the Examiner's statement that one skilled in the art would be motivated to prepare dimers to prevent the emergence of drug-resistant strains is inconsistent with the teachings of the cited references. Specifically, Boeckh teaches that:

> In vitro studies with this combination [of vancomycin and ceftazidime] showed strong synergism and ***prevention of secondary resistance*** to most staphylococci. Boeckh at page 94 (emphasis added).

In contrast, the Renoud-Grappin reference teaches that the dimer approach failed to provide any advantage. Again, one skilled in the art examining the entire teachings of these references would be motivated to explore physical mixtures of antibiotics for the prevention of resistance rather than dimers.

The Examiner has also cited the Staroske reference and stated that:

> [V]ancomycin dimers were also known in the art at the time of filing. Straroske et al discuss both "head-to-head" and head-to-tail" dimers (see Figure 3) and that in "light of recent reports of vancomycin-resistant bacteria" there is a 'strong incentive for the development of more potent antibiotics' (page 4917, bottom). The reference also teaches that dimeric vancomycin compounds exhibit improved antibacterial activity, see for example, page 4918 top. Specifically, the dimers of Staroske et al are linked from the amino terminus of one vancomycin moiety to the carboxy terminus of another (see Scheme 1, page 4919). The reference also contemplates linking of the vancomycin at the vancosamine moiety (see page 4920, last two paragraphs). Final Rejection at page 5.

Again, however, when this reference is examined in its entirety, the actual results provide little or no motivation to prepare dimers. Specifically, although Staroske indicates that certain vancomycin dimers had increased antibacterial activity against a particular strain of resistant bacteria, this reference clearly goes on to say in the same sentence that such dimers "were still insufficiently active for therapeutic use" (Staroske at page 4918, first paragraph). Thus, the only data provided by this reference actually shows that the vancomycin dimers discussed therein were insufficiently active for therapeutic use against the resistant strain of bacteria. In contrast, Boeckh teaches that

physical mixtures of vancomycin and ceftazidime are an "effective regimen" for the treatment of bacterial infections in patients. Based on such teachings, one skilled in the art would be motivated to make *physical mixtures* of antibiotics rather than dimers.

Thus, in summary, the Truett reference teaches that certain antibiotic moieties can be linked together and that such linked antibiotic moieties "can be of value" but also reports that "[a]ll products showed modest inhibition zones." Truett say nothing about the specific combinations of vancomycin and a β-lactam claimed by Appellants. The Renoud-Grappin reference adds to Truett by teaching that the dimer approach failed to increase the inhibitory activity of the disclosed compounds against HIV. The Staroske reference further teaches that the vancomycin dimers discussed therein were insufficiently active for therapeutic use against a particular strain of resistant bacteria. On the other hand, the Boeckh reference teaches that an entirely different approach is therapeutically effective. Specifically, Boeckh teaches that a physical mixture of vancomycin and ceftazidime, i.e., an unlinked mixture of the separate components, is therapeutically effective and shows prevention of secondary resistance to most staphylococci *in vitro*.

Therefore, when taken as a whole, the cited references clearly do not provide any incentive for the skilled artisan to depart from the use of physical mixtures of vancomycin and ceftazidime and prepare dimers of such compounds. In fact, the actual teachings of these references establish just the opposite – based on the teachings of these references, one skilled in the art would recognize that the preparation of dimers requires extra synthetic steps (Truett) and that such dimers do not provide any actual advantage over the monomers (Truett and Renoud-Grappin) but that taking a different approach, i.e., the preparation of physical mixtures, provides compositions that are therapeutically effective (Boeckh). Thus, one skilled in the art would be motivated to prepare *physical mixtures* of antibiotics rather than dimers.

Accordingly, since the cited references clearly do not provide the requisite motivation to one skilled in the art to modify the teachings in a manner that produces Appellants' claimed invention, the Examiner has not established a *prima facie* case of obviousness for Appellants' claimed invention and the rejection of Appellants' claimed subject matter based on these references should be reversed.

3. THE EXAMINER HAS IMPROPERLY DERIVED THE MOTIVATION TO COMBINE REFERENCES FROM APPELLANTS' SPECIFICATION

The Federal Circuit has repeatedly warned that the requisite motivation to establish a *prima facie* case of obviousness must come from the prior art, not applicant's own specification. In determining whether a person of ordinary skill would have been led to a particular combination of references, it is improper simply to "[use] that which the inventor taught against its teacher." *In re Lee*, 277 F.3d 1338, 1343, 61 USPQ2d 1430, 1434 (Fed. Cir. 2002), citing *W.L. Gore v. Garlock, Inc.*, 721 F.2d 1540, 1553, 220 USPQ 303, 312-13 (Fed. Cir. 1983). In particular, using an applicant's disclosure as a blueprint to reconstruct the claimed invention from isolated pieces of the prior art contravenes the statutory mandate of § 103 which requires judging obviousness at the point in time when the invention was made. *See Orthopedic Equip. Co. v. United States*, 702 F.2d 1005, 1012, 217 USPQ 193, 199 (Fed. Cir. 1983) (where the court held that "[i]t is wrong to use the patent in suit as a guide through the maze of prior art references, combining the right references in the right way so as to achieve the result of the claims in suit.").

In the present case, the Examiner has improperly used Appellants' disclosure as a guide to pick the Boeckh reference from the vast array of prior art documents teaching physical mixtures of various antibiotics. By doing so, the Examiner has improperly concluded that one skilled in the art would have been motivated to prepare dimers of vancomycin and ceftazidime when in fact, the cited references relating to dimers provide no reasons to pick vancomycin and ceftazidime from the vast array of known combinations of antibiotics.

Specifically, at the time Appellants' invention was made, many physical mixtures of antibiotics were known in the art. By way of illustration, numerous examples of synergistic antimicrobial combinations are listed in George M. Eliopoulos & Robert C. Moellering, Jr., *Antimicrobial Combinations, in* ANTIBIOTICS IN LABORATORY MEDICINE 330, 373-382 (Victor Lorian ed., 4th ed. 1996) and references cited therein. A copy of this document is attached as APPENDIX B to illustrate the state of the art. In this regard, the Boards' attention is directed to pages 373-82 which list a multitude of synergistic antibiotic combinations, the vast majority of which do not involve vancomycin and a β-lactam antibacterial compound.

In view of this vast array of prior art references relating to synergistic combinations of antibacterial compounds, the Examiner has given no reason why one skilled in the art would select the particular combination of vancomycin and ceftazidime to prepare dimers. In this regard, the Examiner has merely selected the Boeckh reference based on hindsight knowledge of Appellants' claimed invention in order to support her conclusions of obviousness without acknowledging the many other prior art references which teach synergistic combinations of antibiotics. By doing so, the Examiner has improperly used Appellants' disclosure as a guide through this maze of prior art references. Nothing of record supports the conclusion that one skilled in the art would have been led to select vancomycin and ceftazidime for the preparation of dimers in preference to any of the many other known synergistic combinations of antibacterial compounds.

Accordingly, since the Examiner has improperly used hindsight knowledge of Appellants' claimed invention to assemble the cited references, the Examiner has not properly established a *prima facie* case of obviousness for Appellants' claimed invention based on these references.

D. CITED REFERENCES DO NOT PROVIDE A REASONABLE EXPECTATION THAT THE PROPOSED MODIFICATION WILL SUCCEED

To establish a *prima facie* case of obviousness, the Examiner must also show that the proposed modification of the prior art would have had a reasonable expectation of succeeding as determined from the vantage point of the skilled artisan at the time the invention was made. Although obviousness does not require absolute predictability, at least some degree of predictability is required. Thus, evidence showing there was no reasonable expectation of success may support a conclusion of nonobviousness. *See In re Rinehart*, 531 F.2d 1048, 189 USPQ 143 (CCPA 1976). For the following reasons, the Examiner has not shown that the cited references provide the degree of predictability required to establish a *prima facie* case of obviousness and therefore, the rejection of Appellants' claimed subject matter should be reversed.

1. CITED REFERENCES TEACH THE UNPREDICTABILITY OF THE PROPOSED MODIFICATION

The cited references do not provide the necessary degree of predictability because the Renoud-Grappin reference explicitly teaches the failure of the dimer approach and lists numerous factors affecting the predictability of this approach. Thus, the teachings of this reference clearly make the Examiner's stated goal of the proposed modification completely unpredictable.

Specifically, in the present case, the Examiner has stated that the cited references provide the following motivation:

> One of ordinary skill would have been motivated to covalently link vancomycin with ceftazidime to create a broad spectrum antibiotic compound to fight antibiotic resistant strains....Also, the strongest rationale for combining references is a recognition, expressly or impliedly in the prior art or drawn from a convincing line of reasoning based on established scientific principles or legal precedent, that some advantage or expected beneficial result would have been produced by their combination. *In re Sernaker*, 702 F.2d 989, 994-95, 217 USPQ 1, 5-6 (Fed. Cir. 1983). In the instant case, the beneficial result of the combination of references is ***creating a broad spectrum antibiotic compound to fight antibiotic resistant strains***. Final Rejection at page 6 and 7-8 (emphasis added).

Accordingly, the issue is whether one skilled in the art would have had a reasonable expectation that preparing dimers of vancomycin and ceftazidime would create a broad spectrum antibiotic compound to fight antibiotic resistant strains. In this regard, the teachings of Renoud-Grappin clearly establish that one skilled in the art could not have reasonably predicted whether they would succeed in achieving this objective.

Specifically, the Renoud-Grappin reference teaches:

> To prevent the emergence of drug-resistant virus strains, various drugs can be combined....One approach to combination therapy...is the use of heterodimers resulting from the linking of a NNRTI and a NRTI through an appropriate spacer, ***in an attempt*** to combine the inhibitory capacity of these very different classes of molecules. With this aim, we have designed, synthesized and evaluated the anti-HIV-1 activity of several heterodimers.... Renoud-Grappin at page 207 and 208 (emphasis added).

After preparing and testing their dimers, Renoud-Grappin report the following:

> In conclusion, no synergistic antiviral activity and no diminished toxicity were found....Several reasons may explain ***the failure of this heterodimer approach*** to increase the inhibitory activity against HIV....[1.] Some flexibility may be needed to permit penetration of the heterodimer to reach the binding pocket of the RT....[2.] [T]he NNRTI and NRTI parts may be linked to each other on the wrong position; [3.] [I]t also may well be possible that the NNRTI part would not bind the allosteric site in the RT....[4.] [T]he relative inactivity of the heterodimers may be due to poor transport or metabolism of the heterodimer in the host cells used. [5.] The heterodimer could have been hydrolytically cleaved either chemically or enzymatically into the nucleoside part. Renoud-Grappin at page 219 (emphasis added).

Thus, this reference provides both direct evidence of the failure of the dimer approach and lists numerous factors, including flexibility, points of attachment, binding, transport, metabolism, chemical stability and enzymatic stability, that contribute to the unpredictability of this approach. In view of these explicit teachings, one skilled in the art would not have had a reasonable expectation that preparing dimers of vancomycin and ceftazidime would succeed in creating a broad spectrum antibiotic compound to fight antibiotic resistant strains, i.e., the Examiner's stated motivation for combining the cited references.

With regard to the issue of reasonable expectation of success, the Examiner has stated:

> One of ordinary skill would also have had a reasonable expectation of success based on the fact that Staroske et al teaches linking chemistry for vancomycin. Final Rejection at page 6.

Presumably, the Examiner is saying that one skilled in the art would have had a reasonable expectation that dimers of vancomycin and ceftazidime could be prepared successfully based in the linking chemistry taught by the Staroske reference for vancomycin. However, this argument fails to address whether the skilled artisan would have had a reasonable expectation of achieving the Examiner's stated goal for the

modification, i.e., would the skilled artisan have had a reasonable expectation that preparing dimers of vancomycin and ceftazidime would create a broad spectrum antibiotic compound to fight antibiotic resistant strains. For the reasons enumerated above, the teachings of Renoud-Grappin clearly show that one skilled in the art would not have had a reasonable expectation that preparing dimers of vancomycin and ceftazidime would successfully create a broad spectrum antibiotic compound to fight antibiotic resistant strains.

Accordingly, since the cited references clearly do not provide the requisite reasonable expectation of success, the Examiner has not established a *prima facie* case of obviousness for Appellants' claimed invention.

2. OBVIOUS TO TRY IS INSUFFICIENT TO ESTABLISH A PRIMA FACIE CASE OF OBVIOUSNESS

A *prima facie* case of obviousness cannot be based on what the skilled artisan might try or find obvious to try. Rather, the proper test requires determining what the prior art would have led the skilled artisan to do. *See, In re Fine*, 837 F.2d at 1075, 5 USPQ2d at 1599 (Fed. Cir. 1988) ("[W]hether a particular combination might be 'obvious to try' is not a legitimate test of patentability.") (citations omitted).

In the present case, to support her conclusions of obviousness, the Examiner has cited broad, generalized teachings in the references of record which teach possible benefits of dimers or desirable goals or outcomes for an antibacterial research program. For example, the Examiner has stated:

> Truett teaches the "linking of diverse antibiotic moieties via difunctional organic compounds"...The reference teaches that the linkage of two antibiotic moieties can create compounds of new activity...and that "two antibiotics moieties can be linked in which one is known to attack Gram positive bacteria and another to attack Gram negative bacteria"....Renoud-Grappin...describes using heterodimers for combination therapy linked "through an appropriate spacer, in an attempt to combine the inhibitory capacity" of two different classes of molecules....Renoud-Grappin et al also discuss combining different drugs to "prevent the emergence of drug-resistant virus strains"....Staroske et al discuss...that in "light of recent reports of vancomycin-resistant bacteria" there is a "strong incentive for the development of more potent antibiotics".... Final Rejection at pages 3-5.

Such broad teachings regarding the desirability of combating drug-resistant strains of bacteria and developing more potent antibiotics do not provide the requisite motivation necessary to establish a *prima facie* case of obviousness. In particular, it is important to remember that Appellants' claimed invention is directed to novel chemical compounds having a specifically-defined chemical structure. Such broad teachings provide no motivation to prepare the specific compounds that are the subject of Appellants' claims. At best, the cited teachings merely provide an invitation to experiment in the field of antibiotics or dimers; or they make such dimers "obvious to try." However, the courts have long held that "obvious to try" or an "invitation to explore" does not create a *prima facie* case of obviousness.

More specifically, the Federal Circuit has given the following examples of what would constitute an "obvious to try" modification based on the prior art:

> In some cases, what would have been 'obvious to try' would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful. In others, what was 'obvious to try' was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it." *In re O'Farrell*, 853 F.2d 894, 903, 7 U.S.P.Q.2d 1673, 1681 (Fed. Cir. 1988) (citations omitted).

In the present case, the cited references, at best, provide an invitation to explore a new technology or general approach and give only generalized guidance on how to proceed. For example, Renoud-Grappin state the following:

> In conclusion, to obtain better insights into the feasibility of the heterodimer approach to increase the efficacy of compounds against RT, chemists should explore other linkers and attachment sites for these linkers on both non-nucleoside and nucleoside analogues. Renoud-Grappin at page 219.

Clearly, the admonition to "explore other linkers and attachment sites for these linkers" does not provide the necessary motivation to make Appellants' claimed invention, but merely suggests a possible avenue of research to be explored by those skilled in the art.

In response to Appellants' suggestion that the cited reference provide an "invitation to experiment," the Examiner has stated the following:

> The examiner's position is that the references **do** indicate which parameters are critical and **do** provide direction as to which of many possible choices is likely to be successful. For example, Boeckh et al specifically teach combination therapy with vancomycin and ceftazidime and that this combination therapy is used to "cover a broad spectrum of gram positive and gram negative bacteria", Truett teaches the "linking of diverse antibiotic moieties via difunctional organic compounds' and Renoud-Grappin teaches the heterodimer approach for combination therapy, and improvements thereon by exploration of "other linkers and attachment sites for these linkers". Final Rejection at pages 10-11 (emphasis in original).

But again, the Examiner's arguments are based on broad, generalized teachings in the cited references which, at best, provide only general guidance on how to explore a new technology or approach. This is exactly the situation the Federal Circuit characterized as being "obvious to try" in *In re O'Farrell* and as a result, not providing the requisite motivation to establish a *prima facie* case of obviousness.

Accordingly, since the cited references, at best, provide only an "invitation to experiment" or make the claimed invention "obvious to try", the Examiner has not

established a *prima facie* case of obviousness for Appellants' claimed invention.

E. CITED REFERENCES DO NOT TEACH OR SUGGEST ALL THE LIMITATIONS OF CLAIMS 46, 54, 57 AND 58

Additionally, to establish a *prima facie* case of obviousness, the Examiner must show that the prior art reference or combination of references teach or suggest all the limitations of the claims. For the following reasons, the Examiner has not shown that the cited references, alone or when combined, teach or suggest all the limitations of at least Claims 46, 54, 57 and 58.

1. β-LACTAM MOIETIES OF CLAIMS 46, 54, 57 AND 58 ARE NOT TAUGHT OR SUGGESTED BY THE CITED REFERENCES

Appellants' claimed invention comprises a vancomycin moiety covalently linked to specifically-defined β-lactam moieties. For Claims 46, 54, 57 and 58, the Examiner has not established a *prima facie* case of obviousness because none of the cited references, either alone or when combined, teach or suggest the specific β-lactam moieties required by these claims.

Specifically, Truett teaches penicillin- and cephalosporin-type β-lactams and related compounds (*see* Truett at Column 2, lines 16 to Column 3, line 14); and Boeckh teaches ceftazidime, a cephalosporin-type β-lactam (*see* Boeckh at page 92). Renoud-Grappin and Staroske do not teach any β-lactam structures.

In contrast, Appellants' Claim 46 comprises a monobactam-type β-lactam moiety. None of the references relied upon by the Examiner teach or suggest a monobactam-type β-lactam moiety or a dimer of vancomycin and a monobactam-type β-lactam moiety. Thus, even when the cited references are combined, they do not produce or suggest the subject matter of Claim 46.

Similarly, Appellants' Claim 54 comprises a cephalosporin-type β-lactam moiety having a 5-chlorothiazole substituent. None of the references relied upon by the Examiner teach or suggest a cephalosporin-type β-lactam moiety having a 5-chlorothiazole substituent or a dimer of vancomycin and a cephalosporin-type β-lactam moiety having a 5-chlorothiazole substituent. Thus, when the cited references are

combined, they do not produce or suggest the subject matter of Claim 54.

Additionally, Applicants' Claim 57 comprises a cephalosporin-type β-lactam moiety having a specifically defined thioaryl substituent. None of the references relied upon by the Examiner teach or suggest a cephalosporin-type β-lactam moiety having the specifically-defined thioaryl substituent or a dimer of vancomycin and a cephalosporin-type β-lactam moiety having such a thioaryl substituent. Thus, when the cited references are combined, they do not produce or suggest the subject matter of Claim 57.

Finally, Appellants' Claim 58 comprises a cephalosporin-type β-lactam moiety having a specifically-defined thioaryl substituent and a 5-chlorothiazole substituent. None of the references relied upon by the Examiner teach or suggest a cephalosporin-type β-lactam moiety having the specifically defined thioaryl substituent and 5-chlorothiazole substituent or a dimer of vancomycin and such a cephalosporin-type β-lactam moiety. Thus, when the cited references are combined, they do not produce or suggest the subject matter of Claim 58.

Accordingly, since the cited references do not teach or suggest all the limitations of Claims 46, 54, 57 and 58, the Examiner has not met the burden of establishing a *prima facie* case of obviousness for the subject matter of these claims. For this reason, these claims are believed to be separately and independently patentable relative to each other and to the other claims pending in this application.

F. CONCLUSIONS

In summary, the Examiner has not established a *prima facie* case of obviousness for Appellants' claimed invention because she has not shown that the cited references provided the requisite suggestion or incentive that would have motivated the skilled artisan to modify the references in a manner to produce Appellants' claimed invention.

Specifically, there is no structural similarity between Appellants' claimed compounds and the compounds of the cited references, so structural similarity cannot provide the necessary motivation to make Appellants' claimed invention. In the absence of structural similarity, the Examiner has attempted to piece together separate elements taken from the prior art to construct Appellants' claimed subject matter. However, the cited references, when viewed in their entirety, fail to provide the requisite motivation to

combine such elements in a manner that produces Appellants' claimed invention.

More specifically, the cited references teach that extra effort is required to prepare dimers, but the references do not demonstrate any actual advantage to be gained from dimers. Rather than motivating the skill artisan to prepare dimers of antibiotics, the cited references teach that *physical mixtures* of antibiotics, such as vancomycin and ceftazidime, are an "effective regimen" for treating bacterial infections. Thus, the cited references actually provide motivation to the skilled artisan to explore physical mixtures of antibiotics as opposed to dimers.

Moreover, any appearance of motivation to make dimers of vancomycin and ceftazidime created by the cited references diminishes when one realizes that the Examiner has improperly used hindsight knowledge of Appellants' claimed invention as a guide through the maze of prior art references teaching synergistic combinations of antibiotics. Nothing of record supports the conclusion that one skilled in the art would have been led to select vancomycin and ceftazidime for the preparation of dimers in preference to any of the other known synergistic combinations of antibiotics.

Additionally, none of the cited references provide one of ordinary skill in the art with a reasonable expectation that preparing dimers of vancomycin and ceftazidime would create a broad spectrum antibiotic to fight antibiotic resistant strains. To the contrary, the cited references provide direct evidence of the failure of the dimer approach and identify numerous factors that contribute to the unpredictability of this approach. At best, the generalized teachings of the cited references provide an "invitation to explore" the field of antibiotic dimers or make such dimers "obvious to try," but "obvious to try" a general field of research is not the required standard. Therefore, for this reason as well, the Examiner has not proven a *prima facie* case of obviousness for Appellants' claimed invention.

Finally, the cited references, either alone or when combined, do not teach or suggest the specific β-lactam moieties found in Claims 46, 54, 57 and 58. Since the cited references do not teach or suggest all the limitations of these claims, the Examiner has not met the burden of establishing a *prima facie* case of obviousness for the subject matter of these claims.

Accordingly, for the foregoing reasons, Appellants respectfully request that the Board reverse the Examiner's rejection of Claims 41-46, 49-51, 53-55, 57 and 58 as unpatentable under 35 U.S.C. §103(a).

Respectfully submitted,

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APPENDIX A

THE APPEALED CLAIMS

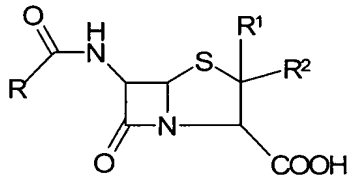
41. A compound of the formula:

L′-X′-L″

or a pharmaceutically acceptable salt thereof; wherein

L′ is a moiety selected from the group consisting of:

(i) a moiety of formula (a):



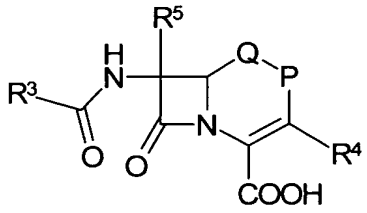
(a)

wherein:

R is selected from the group consisting of substituted alkyl, aryl, aralkyl, and heteroaryl wherein each of said substituents optionally links (a) to the linker via a covalent bond or R is a covalent bond that links (a) to the linker; and

R^1 and R^2 are, independently of each other, alkyl or at least one of R^1 or R^2 is a covalent bond linking (a) to the linker provided that only one of R, R^1 or R^2 links said moiety to said linker;

(ii) a moiety of formula (b):



(b)

wherein:

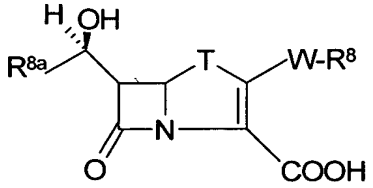
one of P and Q is O, S, or $-CH_2-$ and the other is $-CH_2-$;

R^3 is selected from the group consisting of substituted alkyl, heteroarylalkyl, aralkyl, heterocyclylalkyl, and $-C(R^6)=NOR^7$, wherein R^6 is aryl, heteroaryl, or substituted alkyl and R^7 is alkyl or substituted alkyl and further wherein each of said substituents optionally links (b) to the linker via a covalent bond or R^3 is a covalent bond that links (b) to the linker; and

R^4 is selected from the group consisting of hydrogen, alkyl, alkenyl, substituted alkenyl, substituted alkyl, halo, heteroarylalkyl, heterocyclylalkyl, $-SR^a$ and $-CH_2SR^a$, where R^a is aryl, heteroaryl, heterocyclyl or cycloalkyl wherein each of said substituents optionally links (b) to the linker or R^4 is a covalent bond that links (b) to the linker provided that only one of said R^3 substituents or covalent bond and R^4 substituents or covalent bond links said moiety to said linker; and

R^5 is selected from the group consisting of hydrogen, hydroxy, and alkoxy;

(iii) a moiety of formula (c):



(c)

wherein:

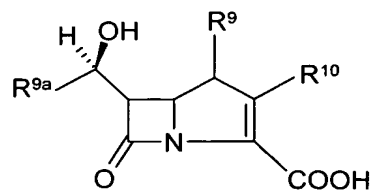
T is S or CH_2,

R^{8a} is alkyl;

W is selected from the group consisting of O, S, $-OCH_2-$, and CH_2; and

R^8 is -(alkylene)-$NHC(R^b)=NH$ where R^b is a covalent bond that links (c) to the linker; or $-W-R^8$ is a covalent bond that links (c) to the linker provided that only one of R^b or $-W-R^8$ binds said moiety to said linker;

(iv) a moiety of formula (d):



(d)

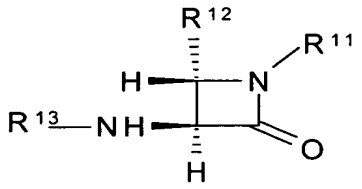
wherein:

R^9 and R^{9a} are alkyl;

R^{10} is selected from the group consisting of hydrogen, alkyl, substituted alkyl, halo, aryl, heteroaryl, heterocyclyl, aralkyl, heteroaralkyl, heterocyclylalkyl and $-CH_2SR^a$, where R^a is aryl, heteroaryl, heterocyclyl or cycloalkyl wherein each of said substituents optionally links (d) to the linker or at least one of R^9 and R^{10} is a covalent bond that links (d) to the linker; or

R^9 and R^{10}, together with the carbon atoms to which they are attached, form an aryl, heteroaryl, cycloalkyl, substituted cycloalkyl, or heterocyclyl ring of from 4 to 7 ring atoms wherein one of the ring atoms optionally links (d) to the linker provided that only one of said substituents, ring atoms, R^9 or R^{10} links said moiety to said linker; and

(v) a moiety of formula (e):



(e)

wherein:

R^{11} is selected from the group consisting of $-SO_3H$ or -(alkylene)-COOH;

R^{12} is selected from the group consisting of alkyl, substituted alkyl, haloalkyl, alkoxy, aryl, aralkyl, heteroaryl, heteroaralkyl, cycloalkyl, substituted cycloalkyl, and heterocyclyl wherein each of said substituents optionally binds (e) to the linker or R^{12} is a covalent bond that links (e) to the linker;

R^{13} is selected from the group consisting of alkyl, acyl, or $-COC(R^{14})=N-OR^{15}$ wherein R^{14} is aryl or heteroaryl which optionally links (e) to the linker, and R^{15} is -(alkylene)-$COOR^{16}$ wherein R^{16} is hydrogen or a covalent bond that optionally links (e) to the linker or R^{13} is a covalent bond that links (e) to the linker provided that only one of R^{12}, R^{13}, R^{14} or R^{15} links said moiety to said linker;

L″ is an optionally substituted vancomycin moiety or an aglycon derivative of an optionally substituted vancomycin moiety, wherein L″ is attached to the linker at a

position selected from the group consisting of the carboxy terminus, the amino terminus, the dihydroxyphenyl ring, the saccharide amino group and the aglycone hydroxy terminus; and

X′ is a linker of the formula:

$$-X^a-Z^a-(Y^a-Z^a)_{m'}-X^a-$$

wherein

m' is an integer of from 0 to 20;

X^a at each separate occurrence is selected from the group consisting of -O-, -S-, -NR′-, -C(O)-, -C(O)O-, -OC(O)-, -C(O)NR′-, -NR′C(O)-, C(S), -C(S)O-, -C(S)NR′-, -NR′C(S)-, and a covalent bond;

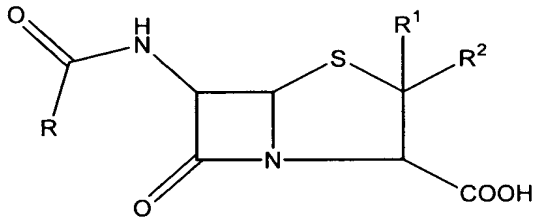
Z^a at each separate occurrence is selected from the group consisting of alkylene, substituted alkylene, cycloalkylene, substituted cylcoalkylene, alkenylene, substituted alkenylene, alkynylene, substituted alkynylene, cycloalkenylene, substituted cycloalkenylene, arylene, heteroarylene, heterocyclene, and a covalent bond;

each Y^a at each separate occurrence is selected from the group consisting of -O-, -C(O)-, -OC(O)-, -C(O)O-, -NR′-, -S(O)n-, -C(O)NR′-, -NR′C(O)-, -NR′C(O)NR′-, -NR′C(S)NR′-, -C(=NR′)-NR′-, -NR′-C(=NR′)-, -OC(O)-NR′-, -NR′-C(O)-O-, -P(O)(OR′)-O-, -O-P(O)(OR′)-, $-S(O)_nCR'R''-$, $-S(O)_n-NR'-$, $-NR'-S(O)_n-$, -S-S-, and a covalent bond; where *n* is 0, 1 or 2; and

R′ and R″ at each separate occurrence are selected from the group consisting of hydrogen, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, alkynyl, substituted alkynyl, aryl, heteroaryl and heterocyclic;

provided that when L″ is a vancomycin moiety attached via its carboxyl group to the linker, then L′ is not a cefalexin moiety attached to the linker via acylation of its α-amino group.

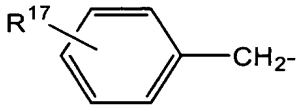
42. The compound of Claim 41, wherein the β-lactam moiety has the formula:

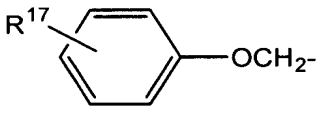


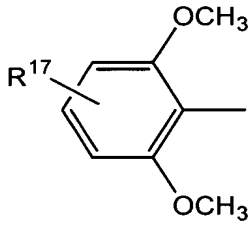
wherein:

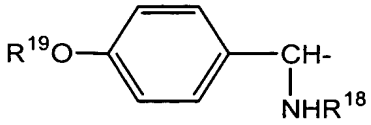
R^1 and R^2 are methyl; and

R is selected from the group consisting of:

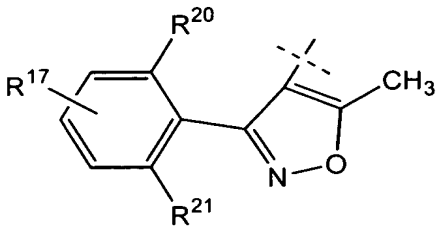
(i) 

(ii) 

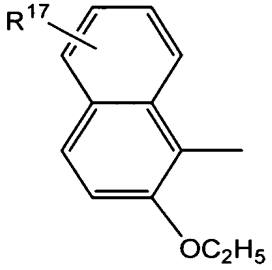
(iii) 

(iv) 

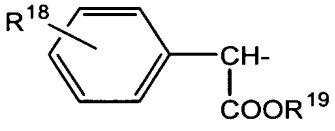
(v)



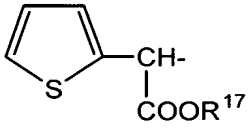
(vi)



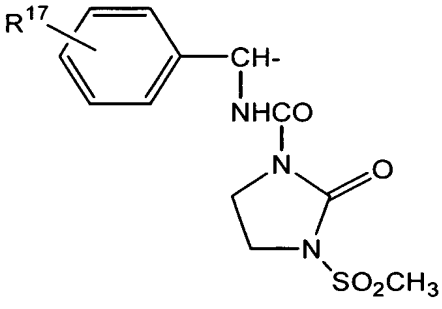
(vii)



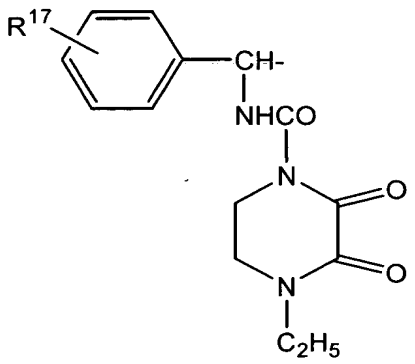
(viii)



(ix)



and

(x) 

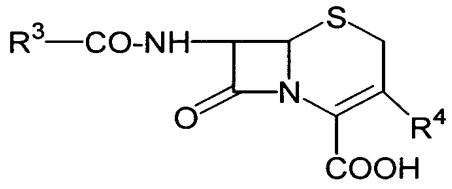
wherein:

R^{17} is a covalent bond that links the β-lactam moiety to a linker;

one of R^{18} and R^{19} is hydrogen and the other is a covalent bond that links the β-lactam moiety to a linker; and

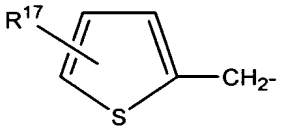
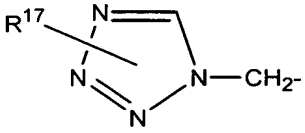
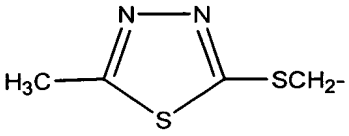
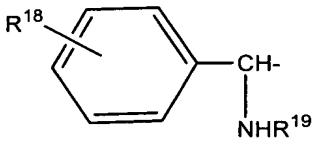
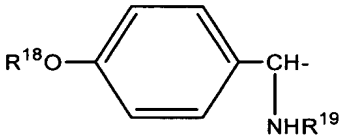
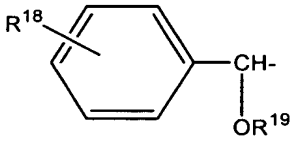
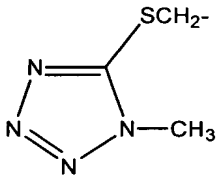
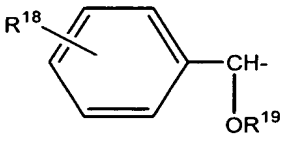
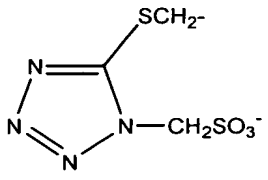
R^{20} and R^{21} are independently selected from the group consisting of hydrogen and chloro.

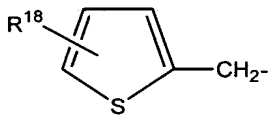
43. (Amended) The compound of Claim 41, wherein L′ is a moiety of the formula:



where:

R^3 and R^4 are selected from the group consisting of:

	R^3	R^4
(i)		$-CH_2OCOCH_3$
(ii)		
(iii)		$-CH_3$
(iv)		$-CH_3$
(v)		
(vi)		

(vii) 

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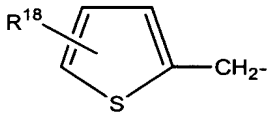
(viii) 

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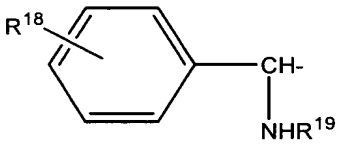
(ix) 

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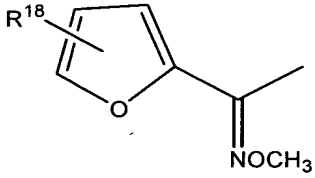
(x) 

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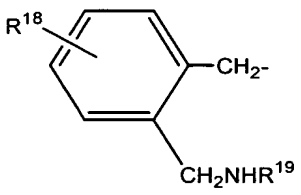
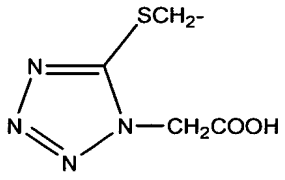
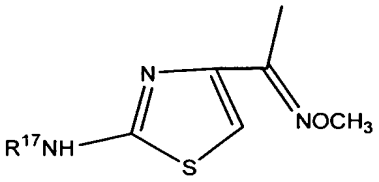
(xi) 



Appendix A-9

(xii) 

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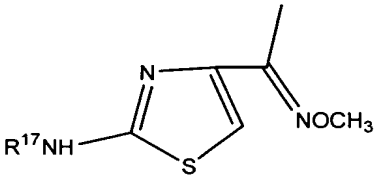
(xiii) 

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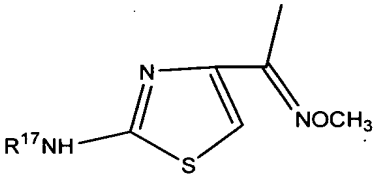
(xiv) 

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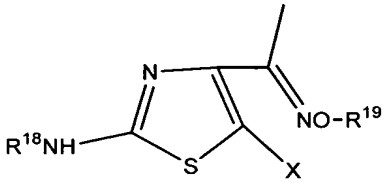
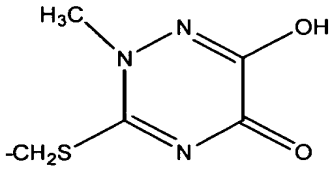
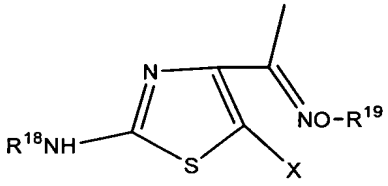
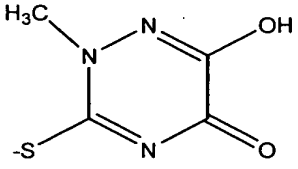
(xv) 



(xvi) 



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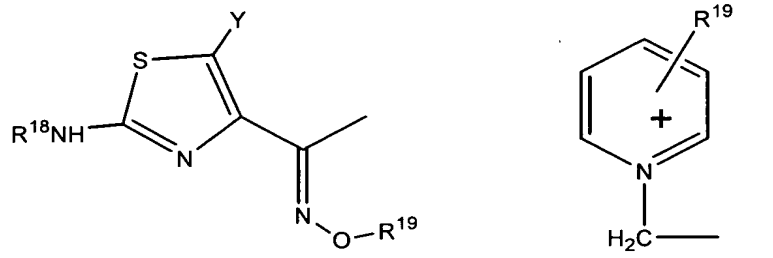

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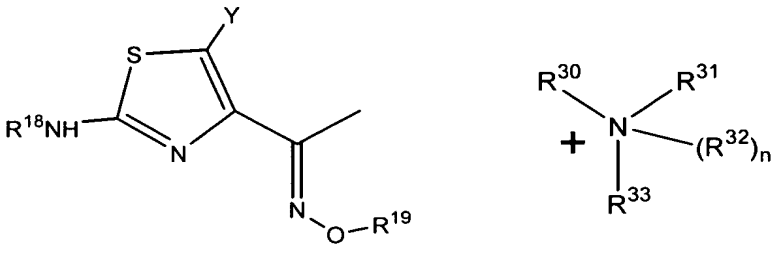

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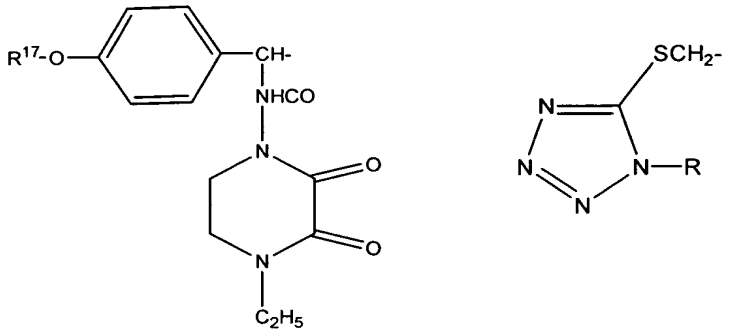

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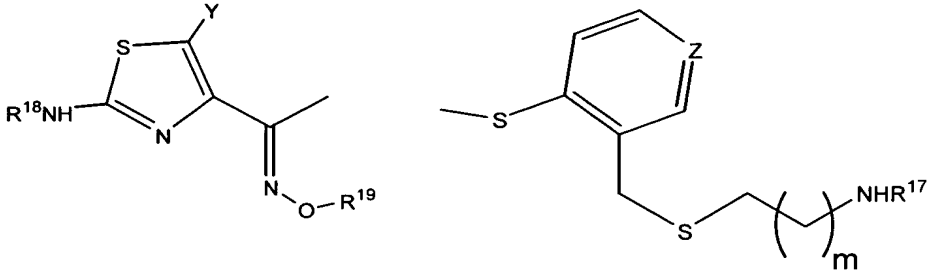

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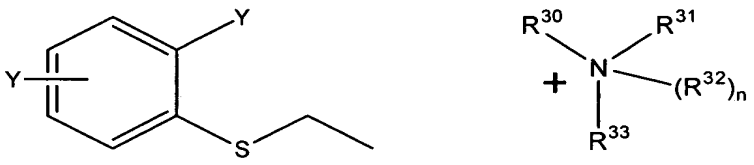


wherein:

R is alkyl;

R^{17} is a covalent bond that links the L′ moiety to the linker;

R^{18} and R^{19} are hydrogen or alkyl;

R^{30} and R^{31} are, independently of each other, hydrogen or alkyl; or together with the nitrogen atom to which they are attached form a heterocycloamino group;

R^{32} is alkyl;

R^{33} is alkylene;

X is halo;

Y is hydrogen or halo;

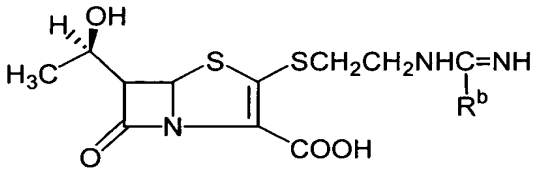
Z is CH or N;

m is an integer from 1 to 5;

n is 0 or 1;

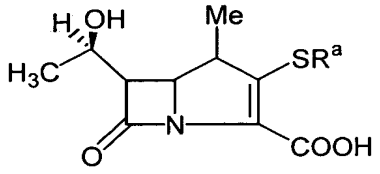
and further wherein one of R^{18}, R^{19}, R^{30}, R^{31}, R^{32} and R^{33} is a covalent bond that links the L′ moiety to the linker.

44. The compound of Claim 41, wherein the β-lactam moiety has the formula:

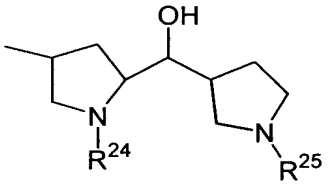


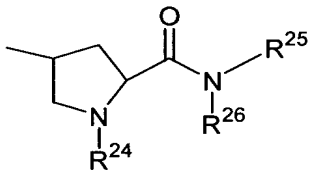
wherein R^b is a covalent bond linking the β-lactam moiety to the linker.

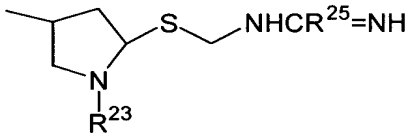
45 The compound of Claim 41, wherein the β-lactam moiety has the formula:

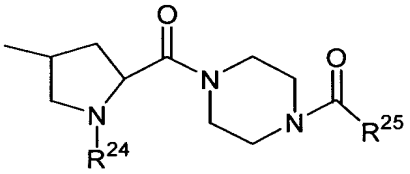


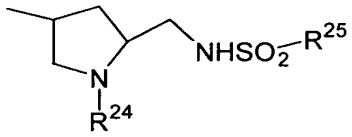
wherein R^a is selected from the group consisting of:

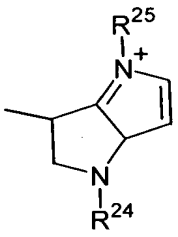
(i) 

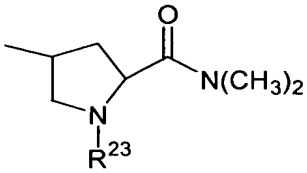
(ii) 

(iii) 

(iv) 

(v) 

(vi) 

(vii) 

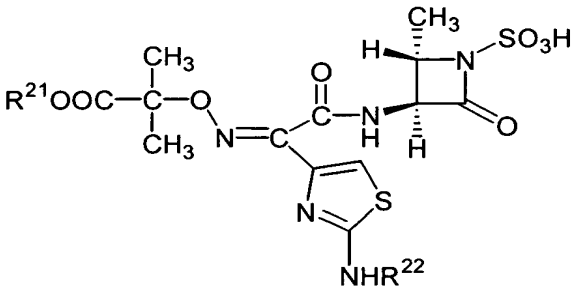
wherein:

R^{23} is a covalent bond that links the β-lactam moiety to the linker;

one of R^{24} and R^{25} is hydrogen, alkyl, substituted alkyl, or aralkyl, and the other is a covalent bond that links the β-lactam moiety to the linker; and

R^{26} is alkyl.

46. The compound of Claim 41, wherein the β-lactam moiety has the formula:



wherein one of R^{21} and R^{22} is hydrogen and the other links the β-lactam moiety to the linker.

49. The compound according to Claim 41 wherein L″ is a vancomycin moiety which is attached to the linker at the saccharide amino group of the vancomycin moiety.

50. The compound according to Claim 41, wherein L″ is a vancomycin moiety which is attached to the linker at the amino terminus of the vancomycin moiety.

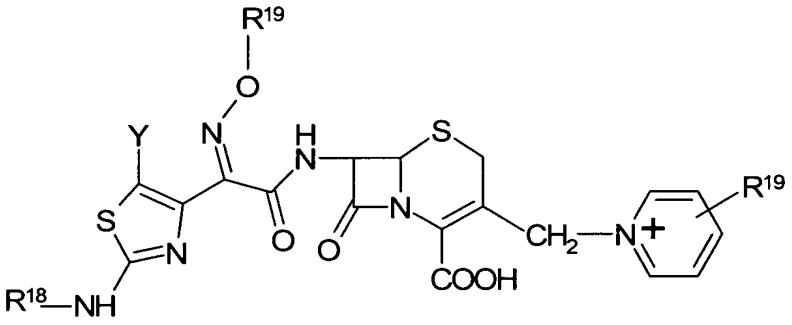
51. The compound according to Claim 41, wherein L″ is a vancomycin moiety which is attached to the linker at the carboxy terminus of the vancomycin moiety.

53. A compound of the formula:

L′-X′-L″

or a pharmaceutically acceptable salt thereof; wherein

L′ is a moiety of the formula:



wherein

Y is selected from the group consisting of hydrogen and halogen;

R^{18} and R^{19} are selected from the group consisting of hydrogen or alkyl provided that one of R^{18} and R^{19} is a covalent bond which links the L′ moiety to the linker; and

L″ is a vancomycin moiety, wherein L″ is attached to the linker at a position selected from the group consisting of the carboxy terminus, the amino terminus, the dihydroxyphenyl ring and the saccharide amino group of the vancomycin moiety; and

X′ is a linker of the formula:

$$-X^a-Z^a-(Y^a-Z^a)_{m'}-X^a-$$

wherein

m' is an integer of from 0 to 20;

X^a at each separate occurrence is selected from the group consisting of -O-, -S-, -NR′-, -C(O)-, -C(O)O-, -OC(O)-, -C(O)NR′-, -NR′C(O)-, C(S), -C(S)O-, -C(S)NR′-, -NR′C(S)-, and a covalent bond;

Z^a at each separate occurrence is selected from the group consisting of alkylene, substituted alkylene, cycloalkylene, substituted cylcoalkylene, alkenylene, substituted alkenylene, alkynylene, substituted alkynylene, cycloalkenylene, substituted cycloalkenylene, arylene, heteroarylene, heterocyclene, and a covalent bond;

each Y^a at each separate occurrence is selected from the group consisting of -O-, -C(O)-, -OC(O)-, -C(O)O-, -NR′-, -S(O)n-, -C(O)NR′-, -NR′C(O)-, -NR′C(O)NR′-, -NR′C(S)NR′-, -C(=NR′)-NR′-, -NR′-C(=NR′)-, -OC(O)-NR′-, -NR′-C(O)-O-, -P(O)(OR′)-O-, -O-P(O)(OR′)-, $-S(O)_nCR'R''-$, $-S(O)_n-NR'-$, $-NR'-S(O)_n-$, -S-S-, and a covalent bond; where *n* is 0, 1 or 2; and

R′ and R″ at each separate occurrence are selected from the group consisting of hydrogen, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, alkynyl, substituted alkynyl, aryl, heteroaryl and heterocyclic.

54. The compound according to Claim 53, wherein Y is halogen.

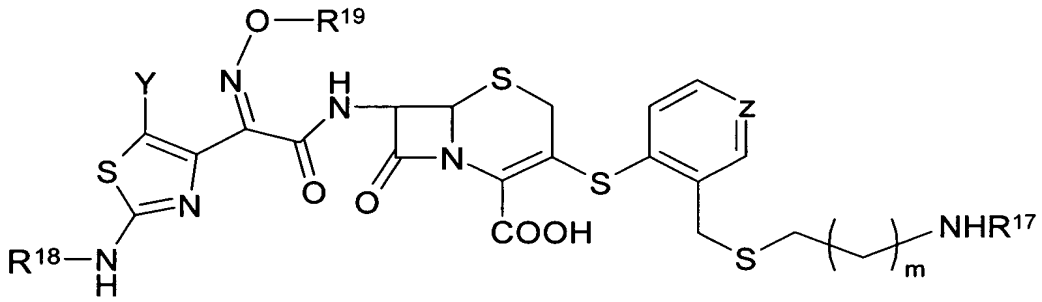
55. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a compound of any of Claims 41-46, 49-51, 53, 54, 57 or 58.

57. A compound of the formula:

L′-X′-L″

or a pharmaceutically acceptable salt thereof; wherein

L′ is a moiety of the formula:



wherein

Y is selected from the group consisting of hydrogen and halogen;

Z is CH or N;

R^{17} is a covalent bond that links the L′ moiety to the linker;

R^{18} and R^{19} are selected from the group consisting of hydrogen or alkyl;

m is an integer from 1 to 5;

L″ is a vancomycin moiety, wherein L″ is attached to the linker at a position selected from the group consisting of the carboxy terminus, the amino terminus, the dihydroxyphenyl ring and the vancosamine amino group of the vancomycin moiety; and

X′ is a linker of the formula:

$$-X^a-Z^a-(Y^a-Z^a)_{m'}-X^a-$$

wherein

m' is an integer of from 0 to 20;

X^a at each separate occurrence is selected from the group consisting of -O-, -S-,

-NR′-, -C(O)-, -C(O)O-, -OC(O)-, -C(O)NR′-, -NR′C(O)-, C(S), -C(S)O-, -C(S)NR′-, -NR′C(S)-, and a covalent bond;

Z^a at each separate occurrence is selected from the group consisting of alkylene, substituted alkylene, cycloalkylene, substituted cylcoalkylene, alkenylene, substituted alkenylene, alkynylene, substituted alkynylene, cycloalkenylene, substituted cycloalkenylene, arylene, heteroarylene, heterocyclene, and a covalent bond;

each Y^a at each separate occurrence is selected from the group consisting of -O-, -C(O)-, -OC(O)-, -C(O)O-, -NR′-, -S(O)n-, -C(O)NR′-, -NR′C(O)-, -NR′C(O)NR′-, -NR′C(S)NR′-, -C(=NR′)-NR′-, -NR′-C(=NR′)-, -OC(O)-NR′-, -NR′-C(O)-O-, -P(O)(OR′)-O-, -O-P(O)(OR′)-, $-S(O)_nCR'R''-$, $-S(O)_n$-NR′-, -NR′-$S(O)_n$-, -S-S-, and a covalent bond; where *n* is 0, 1 or 2; and

R′ and R″ at each separate occurrence are selected from the group consisting of hydrogen, alkyl, substituted alkyl, cycloalkyl, substituted cycloalkyl, alkenyl, substituted alkenyl, cycloalkenyl, substituted cycloalkenyl, alkynyl, substituted alkynyl, aryl, heteroaryl and heterocyclic.

58. The compound according to Claim 57, wherein Y is halogen.

APPENDIX B

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George M. Eliopoulos & Robert C. Moellering, Jr.,

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Accurate indications, adverse reactions and dosage schedules for drugs are provided in this book, but it is possible that they may change. The reader is urged to review the package information data of the manufacturers of the medications mentioned.

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9 Antimicrobial Combinations

GEORGE M. ELIOPOULOS
ROBERT C. MOELLERING, JR.

Antimicrobial combinations are used most frequently to provide broad-spectrum empiric coverage in the treatment of patients who are seriously ill and who may be septicemic. Less frequently, combinations of antimicrobials are chosen because an identified pathogen is resistant to inhibition and/or killing by conventional doses of single antimicrobials but against it the combination may exert the desired antimicrobial activity. In both instances, the clinical outcome may depend on the effects of antimicrobial combinations against individual microorganisms.

In this chapter, we examine the effects of antimicrobial combinations against single microorganisms in vitro. Because specific antimicrobial combinations may be useful against several different microorganisms, we have discussed the various antimicrobial combinations in the text according to their presumed mechanism of interaction. Data on the activities of combinations against specific pathogens are provided in Table 9.7 at the end of the chapter (organized by the genus and species of the pathogen).

Two types of antimicrobial interactions have been excluded from consideration and are not discussed. The first is enhanced effectiveness of an antimicrobial due to another drug that interferes with the elimination or metabolism of the first drug but is not itself an antimicrobial, e.g., the increased penicillin activity produced in vivo by probenecid (which does not have antimicrobial activity at clinically relevant concentrations), which interferes with excretion of penicillin by the kidney (250). The second is the inactivation of one antimicrobial by another, unrelated to the presence of a microorganism, e.g., the inactivation of aminoglycosides by penicillins by a mechanism involving nucleophilic substitution (331, 556). This reaction proceeds readily in vitro (192) if these antimicrobials are stored in the same infusion bottle for prolonged periods (more than 6 to 8 hours) (331). However, it is insignificant in vivo unless the usually rapid excretion of both drugs by the kidney is markedly reduced. For this reason, it is important to measure serum aminoglycoside levels in patients with renal failure who are receiving β-lactam/aminoglycoside combinations (116, 455), to ensure that the desired serum aminoglycoside concentrations are being maintained. We begin by examining the reasons for the use of antimicrobials in combination.

RATIONALES FOR THE USE OF ANTIMICROBIAL COMBINATIONS

Decreased Emergence of Resistant Strains

Antibiotics are sometimes used in combination in an attempt to prevent or delay the in vivo emergence of drug-resistant subpopulations of the pathogenic organism. With the simultaneous use of two or more agents against which bacteria develop resistance by different mechanisms, the probability that colonies will emerge resistant to all of the antimicrobials employed is theoretically very low. This is best illustrated in the treatment of tuberculosis, where simultaneous treatment with multiple drugs clearly reduces the risk of infection with resistant strains (336, 488, 492, 575). This rationale is often discussed for other combinations and may be relevant for combinations containing rifampin, an agent to which many bacteria readily develop resistance if it is used alone. For example, combinations of rifampin with vancomycin and/or other antibiotics have been used successfully in the treatment of prosthetic valve endocarditis due to coagulase-negative staphylococci. Although use of such combinations does in fact appear to suppress the emergence of rifampin resistance in the infecting pathogen, some clinical failures associated with the development of resistant strains have been described (88, 258). This may be due to the emergence of resistant clones in deep foci of infection into which there may be differential penetration of rifampin. Similarly, combination of oxacillin with rifampin suppresses the emergence of *Staphylococcus aureus* populations resistant to the latter, despite the fact that rifampin demonstrates a tendency to antagonize the bactericidal activity of the β-lactam (316). There are also data to support this rationale for the use

of antimicrobial combinations in the treatment of *Pseudomonas* pneumonia. Data from several studies indicate that treatment with a β-lactam alone (ticarcillin, carbenicillin, moxalactam, cefotaxime, piperacillin, or cefoperazone) is associated with the emergence of *Pseudomonas aeruginosa* resistant to β-lactams (152, 206, 233, 440, 485, 495, 552). Although several experimental and clinical studies have produced evidence that combination therapy of *P. aeruginosa* infections with a β-lactam plus an aminoglycoside can reduce the incidence of resistance to either component of the combination (181, 204, 249, 330, 425), results of other studies have not supported this conclusion (39, 49, 90). Nevertheless, in addition to any benefit that may arise from suppression of drug-resistant colonies when infections due to Gram-negative bacteria are treated with combinations of antimicrobials, such combinations may also exhibit synergistic inhibitory or bactericidal activity, as discussed below.

Decreased Dose-Related Toxicity as a Result of Reduced Dosage

Several important antimicrobials have significant dose-related toxicities that seriously limit their use, e.g., chloramphenicol, 5-fluorocytosine (marrow suppression), aminoglycosides, and sulfonamides (nephrotoxicity). Therefore, there are theoretical grounds on which to attempt reduction in the dose of a potentially toxic antimicrobial while using an additional agent to ensure a successful clinical outcome. This approach is exemplified by the former use of sulfonamide combinations (triple sulfonamides) to reduce the incidence of crystalluria with stone formation (301, 302, 334). The success of such regimens—now of historical interest only—was based on the fact that the solubilities of the three sulfonamides (sulfadiazine, sulfamerazine, and sulfamethiazine) are independent of one another, although their antibacterial effects are cumulative (301, 573). At present, there are few antimicrobial combinations that permit a significant reduction in the dose of the toxic antimicrobial (usually an aminoglycoside) without compromising antimicrobial activity. The more recent approach has been to develop newer agents with intrinsically superior safety profiles, which may in many cases be substituted for older, more toxic agents. For example, it may be possible to substitute a β-lactam (or a fluoroquinolone) for an aminoglycoside (for Gram-negative bacillary coverage) in some antibiotic regimens (457).

Polymicrobial Infection

Another important use of antimicrobial combinations is in the treatment of documented or suspected mixed (polymicrobial) infections. In some polymicrobial infections, it may be necessary to treat with agents active against each of several major pathogens. A classic example of this is the rat peritonitis model, in which treatment with agents active against both Enterobacteriaceae and anaerobes is necessary. One drug, such as a cephalosporin or aminoglycoside, protects against early death from the peritonitis and septicemia caused by the former group of organisms. A second drug, such as clindamycin (which is active against anaerobes, particularly *Bacteroides fragilis*), prevents late abscess formation (320). Although such combinations are still frequently employed, the development of newer agents such as imipenem, which is broadly active against both components of mixed infections, now permits successful monotherapy of many polymicrobial infections.

Antimicrobial combinations (or equivalent broad-spectrum single agents) may not always be necessary for the treatment of polymicrobial infections. Several studies have shown penicillin alone to be effective for the treatment of lung abscess, even when both penicillin-susceptible Gram-positive cocci and penicillin-resistant *B. fragilis* are present (35), although later studies suggest that clindamycin (which is usually active against both groups of organisms) may be more effective than penicillin for the treatment of some cases of lung abscess (305). It is important to note that the polymicrobial nature of many supposedly mixed infections is defined poorly, if at all, and the patient often receives empirical broad-spectrum coverage for an undefined infection. In evaluating such combinations, one would seek to determine whether the drugs being used together potentially interfere with one another in vitro (see the individual sections below on antimicrobial antagonism and specific combinations).

Antimicrobial Synergism

Antimicrobial combinations were first used to treat patients early in the antibiotic era when it became apparent that not all infections responded to treatment with sulfonamides, penicillin, or streptomycin used alone. Despite its obvious empiricism, some of this early experience was remarkably successful, as illustrated by the discovery and study of the synergistic bactericidal activity of penicillin and streptomycin against enterococci by Hunter (239, 240) and Jawetz et al. (246–248). This use of antimicrobial combinations to achieve in vitro activity and clinical efficacy against organisms resistant to inhibition and/or killing by acceptable (i.e., nontoxic) concentrations of single agents continues to be the subject of intensive investigation and a matter of great clinical relevance.

There is now a considerable body of literature pertaining to the role of antimicrobial synergism in the treatment of infections due to a wide variety of Gram-positive and Gram-negative organisms. However, in addition to the well-documented value of synergistic bactericidal combinations of penicillin (or ampicillin or vancomycin) plus streptomycin or gentamicin for the treatment of enterococcal endocarditis (367, 368, 571) and the obvious merit of fixed combinations such as trimethoprim (TMP)/sulfamethoxazole (SMZ) in selected situations (72), there are surprisingly few circumstances in which in vitro documentation of antimicrobial synergism has been highly

Table 9.1
Quantitative Definitions of Results with Antimicrobial Combinations[a]

	Checkerboard	Kinetic Methods
Assumptions	Assumes linear (or identical) dose-response curves for all drugs tested	Do not assume linear or identical dose-response curves
Additivity	The result with two drugs is equal to the sum of the results for each of the drugs used separately, as defined by $FIC_A + FIC_B = 1.0$	The result with two drugs is equal to the combined activity of each of the drugs used separately, as defined by $g_{(A+B)} = \frac{g_{(A)} \times g_{(B)}}{g_0}$
Autonomy (indifference)	The result with two drugs does not significantly differ from the result with the most effective drug alone $FIC_A + FIC_B = FIC_A$ or FIC_B	The result with two drugs equals the effect of the more active drug used alone $g_{(A+B)} = g_{(A)}$ or $g_{(B)}$
Antagonism	The result with two drugs is significantly less than the additive response $FIC_A + FIC_B > 1.0$	The result with two drugs is significantly less than the autonomous response $g_{(A+B)} > g_{(A)}$ or $g_{(B)}$
Synergism	The result with two drugs is significantly greater than the additive response $FIC_A + FIC_B \leq 0.5$	The result with two drugs is significantly greater than the additive response $g_{(A+B)} < \frac{g_{(A)} \times g_{(B)}}{g_0}$

Modified from King, Schlessinger, and Krogstad (Ref. 266) with permission of the *Reviews of Infectious Diseases* and the University of Chicago Press. (© 1981, The University of Chicago.)
[a]FIC_A and FIC_B are the fractional inhibitory concentrations of drugs A and B (the amount of each drug necessary to inhibit growth when combined with the other, divided by its minimum inhibitory concentration, defined in Table 9.4). g_0, g_A, g_B, and $g_{(A+B)}$ represent the growth constants observed in the absence of antimicrobials (g_0), with drug A alone (g_A), drug B alone (g_B), and with both drugs A and B (g_{A+B}).

predictive of superior clinical efficacy. Potential advantages of synergistic combinations in the treatment of infections due to Gram-negative bacilli (particularly *P. aeruginosa*) have been observed primarily in neutropenic patients (8, 91, 122, 294). Even among this group, however, it remains uncertain whether such apparent benefits accrue from synergistic interactions per se or arise instead from the superior serum bactericidal activity of certain combinations, relative to those attainable with individual agents. The significance of this admittedly subtle distinction lies in the fact that serum bactericidal titers against various Gram-negative pathogens that can be attained with several of the newer β-lactam antibiotics may actually exceed those achieved with combinations of aminoglycosides with older β-lactams (551). (These issues are discussed further in Reference 139.)

Further stimulus for study of antimicrobial interactions derives from the increasing awareness that certain combinations of agents may, in fact, yield antagonistic effects. A classic example of such effects emerges from studies by Lepper and Dowling (304), who found that the addition of chlortetracycline markedly reduced the survival rate of children treated with penicillin for pneumococcal meningitis from 79% to 21%. More relevant to recent practice, however, are observations documenting the potential for antagonistic interactions between two β-lactam antibiotics used in combination against Gram-negative bacteria possessing inducible (derepressible), chromosomally mediated β-lactamases (194, 473, 474, 476–479).

Based on the preceding comments, it should be apparent that situations arise requiring verification of synergistic interactions between two antimicrobials against an infecting microorganism. Equally important, in vitro studies may be utilized to exclude antagonistic interactions between antibiotics when these cannot be accurately anticipated from general knowledge of drug characteristics. Several methods have been developed to assess such potential drug interactions either quantitatively or qualitatively.

Definitions of Antimicrobial Interactions In Vitro

Despite differences in experimental methods and the criteria used to define quantitatively the results of antimicrobial combinations, there is general agreement on qualitative definitions of synergism and antagonism. Synergism is a positive interaction; the combined effect of the drugs being examined is significantly greater than the expected result, based on their independent effects

when the drugs are used separately (Table 9.1). Antagonism is a negative interaction; the combined effect of the drugs being examined is significantly less than their independent effects when they are tested separately.

Many of the problems involved in assessing antimicrobial combinations result from uncertainty about the expected result with combinations in which there is no significant interaction between the antimicrobials being tested, whether that result should be described as additivity, indifference, or autonomy. Additivity is the basis of the checkerboard system and assumes that the result observed with more than one drug should be the sum of the separate effects of the drugs being tested if those drugs do not interact with one another. Autonomy (or indifference) is based on the idea that only one metabolic pathway can be growth rate-limiting for an organism at a time (266). Based on this observation, autonomy suggests that the combined effect of drugs that do not interact with one another should be simply the effect of the more (most) active drug alone (Table 9.1) (265, 266).

LABORATORY METHODS USED TO ASSESS THE ACTIVITY OF ANTIMICROBIAL COMBINATIONS

Ch ckerboard

The checkerboard (or chessboard) method is the technique that has been used most frequently to assess antimicrobial combinations in vitro (179, 463), presumably because (*a*) its rationale is easy to understand, (*b*) the mathematics necessary to calculate and interpret the results are simple, (*c*) it can be readily performed in clinical laboratories using microdilution systems that are obtainable commercially, and (*d*) it has been the technique most frequently used in studies that have suggested an advantage of synergistic therapy in the treatment of neutropenic patients with Gram-negative septicemia (269, 294). The term "checkerboard" refers to the pattern (of tubes or microtiter wells) formed by multiple dilutions of the two antimicrobials being tested, in concentrations equal to, above, and below their minimal inhibitory concentrations (MICs) (for a definition of this term, see Chapter 2) for the organisms being tested (Fig. 9.1).

The concentrations tested for each antimicrobial typically range from four or five dilutions below the MIC to twice the MIC (or higher if antagonism is suspected), using twofold dilutions of each antimicrobial. Also included is a row (or column) of tubes or microtiter wells for each drug without any antimicrobial. Thus, the checkerboard consists of columns in which each tube (or well) contains the same amount of the drug (drug A) being diluted along the *x* axis and rows in which each tube (or well) contains the same amount of the drug (drug B) being diluted on the *y* axis (Fig. 9.1). The result is that each square in the checkerboard (which represents one tube or well) contains a unique combination of the two drugs being tested. (Although we have expressed the antimicrobial concentrations as multiples or fractions of the MIC in Fig. 9.1, they are usually expressed in micrograms per milliliter.) The dilutions of the antimicrobials being tested are usually performed in Mueller-Hinton broth or another suitable broth for bacterial studies, so that the drug-containing solutions can be mixed with drug-free medium to produce the final concentrations designated on the diagram (179, 463). As noted below (in "Modifications of the Checkerboard Technique"), this technique may be performed with liquid or semisolid (agar) media, with microtiter trays rather than racks of test tubes, using other than twofold dilutions, using more than two drugs, and with organisms other than bacteria.

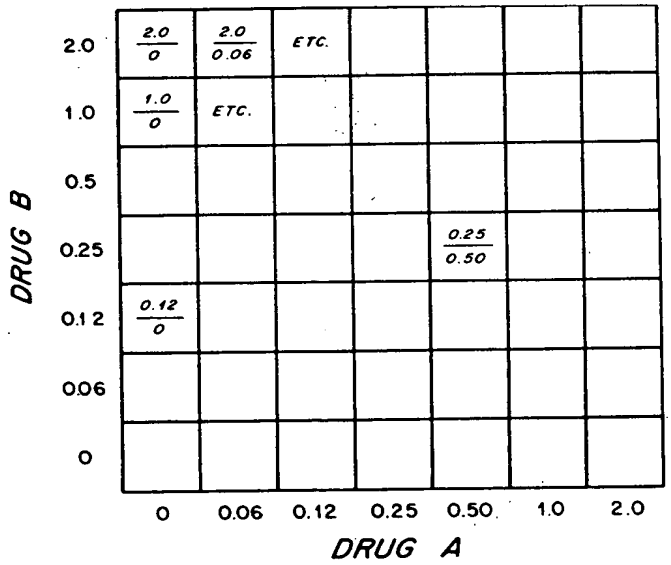


Figure 9.1. Checkerboard technique. In the checkerboard, serial dilutions of two drugs are performed using drug concentrations proportional to the MICs of the drugs being tested. (Although usually expressed in micrograms per milliliter, the concentrations of drugs A and B are expressed here as multiples of the MIC.)

Broth Method

With test tubes, it is convenient to use a final volume of 1.0 mL in each tube: 0.5 mL of broth containing antimicrobials (0.25 mL of broth for each drug if two drugs are being tested) and 0.5 mL of broth containing a suspension of the organism to be tested. Because the final volume (1.0 mL) is four times as great as the volume of broth for each antimicrobial (0.25 mL) with this method, the antimicrobial concentrations used in the initial (stock) solutions should be fourfold greater than the desired final concentrations. For example, if the MIC of drug A (Fig. 9.1) is 1.0 μg/mL, the concentration of drug A in the solution added to the tubes in the far right column of the figure should be 8.0 μg/mL to produce a final concentration of 2.0 μg/mL, which is twice the MIC of the organism being tested. Similarly, the inoculum of the bacterial suspension added to each tube (in 0.5 mL of broth) should be approximately 2×10^5 colony-forming units (CFU)/mL after the addition of an equal volume of the antimicrobial solutions. If broth dilution studies are being performed to assess bactericidal activity by culturing a 10-μL sample for more than 99.9% killing, a final inoculum between 3 and 10×10^5 CFU/mL ensures greater accuracy of test results (424).

With a series of antimicrobial solutions containing four times the desired final concentrations, one can then produce the desired range of drug concentrations. Instead of making a separate set of dilutions for each tube, most workers find it more convenient to prepare larger volumes of the working (4×) antimicrobial solutions and to add an aliquot of those solutions to each tube in the appropriate row or column (as noted in Table 9.2). For example, one would add 0.25 mL of the 8.0 μg/mL solution of drug A to each tube in the far right column of Figure 9.1. Similarly, if one were using an automated device to prepare a series of microtiter plates for checkerboard testing, the 8.0 μg/mL solution of drug A would be added to each of the tubes in the far right column of the machine. Although this dilution scheme is easy to understand, it can be readily modified when it is desirable to work with different final or transfer volumes of antimicrobials or bacteria.

Although the dilutions used in the checkerboard are exponential (by powers of two) (Fig. 9.1), the results of checkerboard testing are interpreted by the pattern they form on the isobologram (which converts those data from an exponential [logarithmic] scale to an arithmetic one) (Fig. 9.2) (266). In Figure 9.2, *A* to *C*, experimental results are shown (shading indicates observable growth) that correspond to additive (Fig. 9.2*A*), synergistic (Fig. 9.2*B*), and antagonistic (Fig. 9.2*C*) interactions, as diagrammed in the isobolograms in Figure 9.2, *D*, *E*, and *F*, respectively. The isobologram is constructed as follows. For each concentration of drug along the *x* axis (plotted as the *x* coordinate), the lowest concentration of the drug diluted along the *y* axis that inhibits growth in the column of tubes is taken as the *y* coordinate of this plot. An isobologram is constructed by connecting the series of coordinate points generated for each drug combination.

Agar Dilution Method

The principles of the techniques outlined above can also be adapted for use in an agar dilution system. This method may be advantageous when a large number of strains are to be tested against a limited number of antibiotic combinations. The volume of the bacterial inoculum does not enter into the calculations used to determine the dilution of the initial antimicrobial stock solutions with this method, because the inoculum is applied to the surface of the agar plate. Therefore, some workers mix equal parts of molten agar containing each of the drugs being tested, using stock concentrations twice the desired final concentrations. If antimicrobials are added to the agar after it has been autoclaved, the agar should first be allowed to cool to 50°C to 55°C in a water-bath to prevent loss of activity with antimicrobials susceptible to heat (539). As discussed for broth testing, serial twofold dilutions can then be carried out with plain agar to obtain the desired range of drug concentrations. However, serial dilutions in agar are tedious and difficult to perform accurately. Therefore, it is often more convenient and more accurate first to dilute the antimicrobials in broth and then to add that broth (containing antimicrobials) to the molten agar. In order to maintain the desired concentrations of both agar and antimicrobials, the volume of broth (containing antimicrobial) added to the agar should be small (i.e., ≤5% of the total volume). Thus, the concentration of the antimicrobial in the broth added to the agar suspension should be ≥20 times the desired final antimicrobial concentration in the agar (Table 9.3).

After the agar plates have been poured and allowed to cool and dry, the bacteria to be tested can be applied to the agar surface with a replicating device designed to deliver a standard inoculum (usually approximately 10^4 CFU) (560). The surface of the agar plate must be dry before the inoculum is applied, because a watery film

Table 9.2
Dilution of an Antimicrobial for Checkerboard Testing in Broth[a]

Desired final concentration of drug	0	0.06	0.12	0.25	0.50	1.0	2.0
Concentration of drug in stock solution	0	0.25	0.50	1.0	2.0	4.0	8.0
Volume of stock solution per tube (mL)	0.25	0.25	0.25	0.25	0.25	0.25	0.25

[a]For the purposes of this table, concentrations of drug are expressed in multiples of the MIC. Each tube contains a total volume of 1.0 mL: 0.25 mL of drug A, 0.25 mL of drug B, and 0.5 mL of bacterial inoculum.

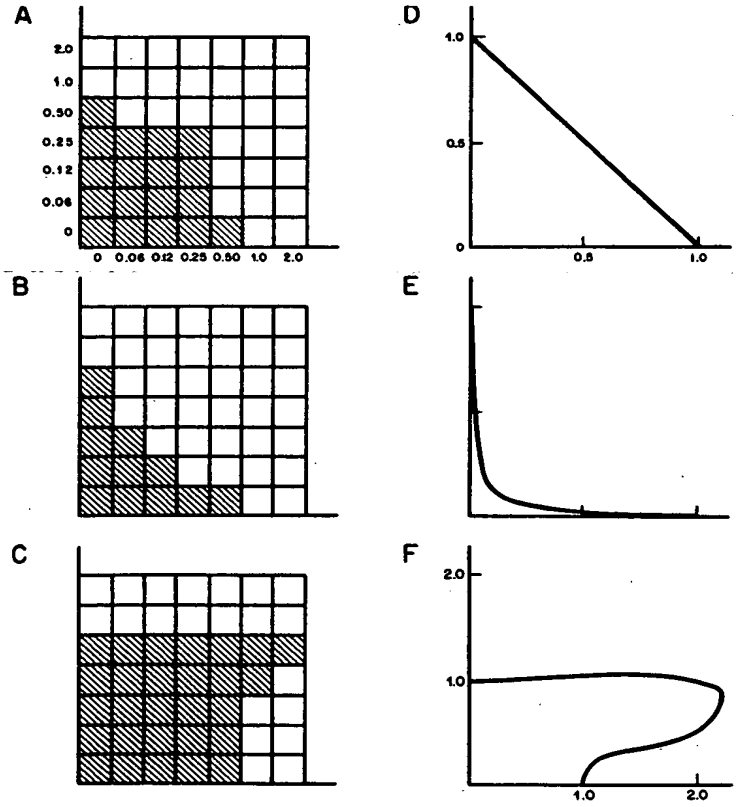


Figure 9.2. Assessment of antimicrobial combinations with the checkerboard method. **A**, **B**, and **C**. Results of testing combinations of two drugs (diluted in geometric twofold increments along the *x* and *y* axes, as in Fig. 9.1). *Shading*, visible growth. Concentrations are expressed as multiples of the MIC. **D**, **E**, and **F**. Isobolograms (plotted on an arithmetic scale) that represent the results of checkerboards shown in **A**, **B**, and **C**, respectively. **A** and **D**. Additive effect. **B** and **E**. Synergism. **C** and **F**. Antagonism.

Table 9.3
Dilution of an Antimicrobial for Checkerboard Testing in Agar[a]

Final concentration of antimicrobial desired	0	0.06	0.12	0.25	0.50	1.0	2.0
Concentration of antimicrobial stock solution	0	1.25	2.5	5.0	10.0	20.0	40.0
Volume of stock solution added to 19.0 mL of agar (mL)	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a]Concentrations of drug are expressed in multiples of the MIC. For testing two drugs, each 20-mL aliquot for a 100-mm agar plate contains 1.0 mL of the appropriate stock solution of drug A, 1.0 mL of drug B, and 18.0 mL of agar.

may permit one or two resistant organisms to spread over the entire agar surface. Condensation on the agar surface is often a concern because agar plates are frequently kept refrigerated after their initial preparation (because some antimicrobial agents remain stable in agar for at least one week at 4°C) (461). Therefore, agar plates should be removed from the refrigerator several hours before their expected use, to permit them to reach room temperature. Plates with persistent condensation should be dried, with their lids ajar, at 35°C to 37°C for 1 to 2 hours before they are inoculated.

Inoculum

Use of the correct inoculum is critical for the accuracy and reproducibility of checkerboard testing, as it is for most susceptibility testing. An excessively large inoculum may result in a falsely high estimate of the MIC due to the "inoculum effect," which usually reflects either the inactivation of β-lactams by β-lactamase or the selection of resistant mutants, although in many cases the mechanisms of this effect are poorly understood. Conversely, an excessively small inoculum may lead to a falsely low estimate of the MIC.

For bacterial studies, the inoculum can be standardized by matching the turbidity of the culture to the 0.5 McFarland standard (386). (This is produced by mixing 0.5 mL of 0.048 mol/liter $BaCl_2$ [1.175%, w/v, $BaCl_2 \cdot 2H_2O$] with 99.5 mL of 0.36 N H_2SO_4 and should be protected from light.) This produces an inoculating suspension of approximately 10^8 CFU/mL, which is then diluted in fresh broth to achieve final inocula of approximately 10^5 CFU/mL for broth dilution studies using MIC endpoints or to achieve larger final inocula if min-

imal bactericidal concentration (MBC) endpoints are to be assessed (424). For agar dilution studies the initial 0.5 McFarland inoculum is diluted approximately 1:10 in fresh broth, and 1 to 2 μL of that suspension are applied to the surface of an agar plate with a calibrated wire loop or a Steers replicator (514), for an inoculum of approximately 10^4 CFU/spot (560). The National Committee for Clinical Laboratory Standards recommends that anaerobic bacteria be tested at an inoculum 10-fold greater than that for aerobes (385). In either case, it is prudent to verify the actual inoculum density by determining colony counts for representative strains of the species to be tested.

Modifications of the Checkerboard Technique

Use of Microtiter Trays. Large amounts of antimicrobials and media are required for standard checkerboard testing with racks of test tubes containing mixtures of both drugs being tested. Therefore, many workers prefer microtiter trays because they are less cumbersome and require smaller quantities of antimicrobials and broth. In addition, automated equipment can be used to prepare dilutions of the two drugs being tested and to deliver the inoculum to each microtiter well.

Dilutions may be made with hand-held or automated microdiluters. Similarly, the transfer plate technique may be used (either manually or with automated microdiluters) to prepare dilutions of a second drug (129). The contents of the transfer plate are then transferred to a standard microdilution plate containing appropriate concentrations of the first antimicrobial, thus creating the desired two-dimensional matrix of drug concentrations. Although smaller volumes are used with this technique than with the standard test tube (macro) checkerboard technique, the information produced is basically similar. However, there are several potential problems with microtiter checkerboard testing that are not encountered when using the macro (test tube) method.

The first problem is evaporation. Because the volumes in each microtiter well are small (100 to 200 μL), modest amounts of evaporation may significantly increase the concentrations of the antimicrobials being tested and thus produce artifactually low MICs (and/or MBCs). This can best be prevented by using plastic adhesive sheets or lids available through the manufacturers of these plates. If the results suggest this possibility (i.e., if the MICs and/or MBCs are lower than expected), aliquots of broth should be saved from several microtiter wells to determine whether the antimicrobial concentrations in those wells were greater than expected.

The second problem is anaerobiosis. The experience of many investigators suggests that these plastic sheets or lids (mentioned above) do not inhibit the growth of strict aerobes. However, the best control to exclude this possibility is luxuriant growth of the test organism in the well(s) without antimicrobial. (Several manufacturers have suggested punching small holes in the plastic sheets over each microtiter well with a pin. This obviates the possibility of anaerobic conditions and also decreases the condensation on the undersurface of the plastic sheet.)

The third problem is surface electricity. Static electricity may accumulate if these plates are not adequately grounded and may interfere with effective dilution of the antimicrobials. Wiping the bottom of the plate with a moist cloth is usually sufficient to resolve this problem.

Variable Dilutions. Although twofold dilutions have been used traditionally with this method (in both its macro- and microtiter versions), they may be insufficiently sensitive for many organisms, especially if the breakpoint is at the upper end of the clinically acceptable range of concentrations for a toxic antimicrobial, such as an aminoglycoside. For this reason, it is often helpful to use less than twofold dilutions (for the whole series of dilutions or at least for those of greatest interest) (265). From a mathematical point of view, the smallest dilution increments provide the greatest precision for subsequent evaluation of the results (see below). Giant checkerboards can be created by combining a series of smaller matrices, each using different dilutions of the two drugs in an attempt to increase precision of endpoint determinations (237). This technique involves considerable work and does not fully circumvent limitations inherent in the standard twofold dilution method.

Testing More than Two Drugs. Using the same dilution scheme, it is also possible to create a three-dimensional (or *n*-dimensional) lattice to study combinations of three (or *n*) drugs against a single pathogen (Fig. 9.3) (42–44). This strategy was developed by Berenbaum, who also used it to quantitate the results with the fractional inhibitory concentration (FIC) index (see below).

Testing Organisms Other than Bacteria. Because bacteria are relatively easy to grow in the laboratory and because antibacterial drugs are often used in combination with one another, most checkerboard testing has been performed with antibacterial agents. However, the same approach has also been used to examine the activity of other antimicrobial combinations against nonbacterial pathogens such as parasites and fungi (327, 405). Measures of antimicrobial action used in these studies have included morphologic criteria (e.g., reductions in parasite counts as measured by Giemsa-stained smears for antimalarials), isotope uptake (the reduction of [^{3}H]hypoxanthine or [^{3}H]thymidine uptake for antiparasitic and antiviral agents) (83, 236, 432, 521), and quantitative cultures (of fungi and viruses) (236, 522). From a conceptual point of view, the nature of the testing scheme is the central issue, not the specific microorganism being tested.

Definition of the Endpoint. Most checkerboard tests are read by examining the tubes (or wells of the microtiter plate) for evidence of visible growth after 16 to 20 hours of incubation at 35°C to 37°C. However, bactericidal data may be obtained if the tubes (or wells)

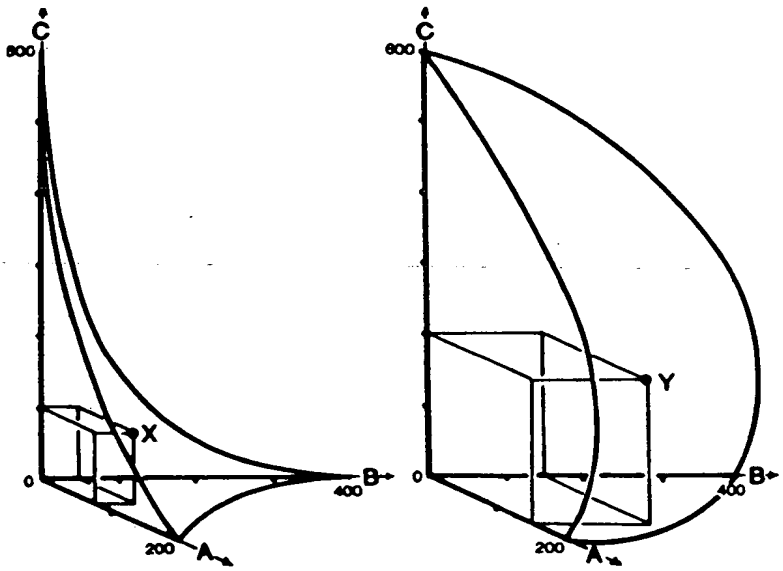


Figure 9.3. Three-dimensional isobolograms used to depict the results of combinations with three antimicrobials. In these diagrams, points corresponding to synergism are on concave surfaces (*left*) and those corresponding to antagonism are on convex surfaces (*right*). (Reproduced from Berenbaum [42], with permission.)

without visible evidence of bacterial growth are sampled to determine the concentrations of the combination producing ≥99.9% killing (the MBC, which is defined and discussed in Chapter 2). Similarly, some investigators have modified the determination (and the definition) of the MIC by using 50% reduction in the optical density produced by bacterial growth to replace visual inspection. They believe that this measure (the 50% inhibitory concentration), when assessed with a spectrophotometer, is more accurate than the traditional MIC (2, 419), although no comparative studies have addressed this question.

Interpretation of the Results

As noted above, most checkerboard (broth and agar dilution) studies are read after 16 to 20 hours of incubation at 35°C to 37°C, although studies of rapidly growing pathogens may frequently be read after 6 to 8 hours of incubation, whereas slowly growing organisms may require longer incubation. In interpreting the results, the critical question is usually whether an apparently synergistic combination is significantly below the additive line of the isobologram. Because the margin of error in these studies is plus or minus one dilution, a combination should be at least two dilutions below the additive line of the isobologram to be significantly synergistic or at least two dilutions above the additive line to be significantly antagonistic.

Because the determination of synergism often depends on the tubes (wells) near the midpoint of the isobologram, many workers have examined only the MIC (or MBC) and one lower concentration of each drug alone, in addition to a group of comparably increasing concentrations of both drugs (in a ratio of their MICs or MBCs), beginning from the control (without drug A or B) and progressing toward the point with x = one half the MIC of drug A and y = one half the MIC of drug B. This scheme (Fig. 9.4) substantially reduces the number of tubes and dilutions necessary to perform the test, while retaining sufficient discrimination at the midpoint to define synergism in most cases.

The fractional inhibitory concentration index of Elion et al. (137) can be viewed as a mathematical restatement of the isobologram (Table 9.4) and is the method most commonly used in the literature to report the results of studies with antimicrobial combinations. In this method, the FIC for each drug is derived by dividing the concentration of that drug necessary to inhibit growth in a given row or column by the MIC of the test organism for that drug alone. The FIC index is then calculated by summing the separate FICs for each of the drugs present in that tube (or well) (Table 9.4).

Although each antimicrobial combination can produce a series of FIC index values if one calculates separate FIC indexes for each row or column in the isobologram, most investigators use the value obtained for equally effective concentrations of the two drugs being tested (i.e., the result obtained by examining combinations in which the ratios of the concentrations of the two antimicrobials are equal to the ratios of their MICs, as in the 45° line in Fig. 9.4) as representative of that combination. (Although this approach simplifies the calculations, it may be misleading because the ratios of the two drugs may not remain constant in vivo, even if they are administered in doses proportional to their MICs.) With this method, synergism is defined as a FIC index of ≤0.5 and additivity as a FIC index of 1.0; antagonism has been often defined as a FIC index of ≥2.0 (179, 463). More recent criteria suggest that a FIC index of more than 4 should be applied to definitions of antagonism to account

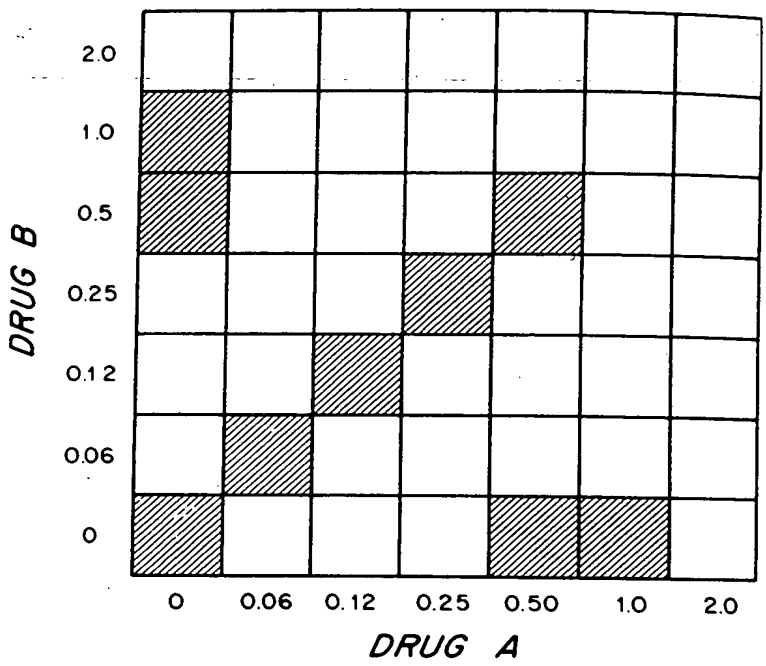


Figure 9.4. Simplified checkerboard testing of antimicrobial combinations. With this approach, the number of combinations tested may be substantially reduced (e.g., in this case to only 7 of 49 combinations). *Hatching*, combinations tested.

Table 9.4
Calculation of the Fractional Inhibitory Concentration (FIC) Index for Combinations of Two Antimicrobials[a]

$$\frac{(A)}{(MIC_A)} + \frac{(B)}{(MIC_B)} = FIC_A + FIC_B = \text{FIC index}$$

[a](A) is the concentration of drug A in a tube that is the lowest inhibitory concentration in its row. (MIC_A) is the MIC of the organism to drug A alone. FIC_A is the fractional inhibitory concentration of drug A. (B), (MIC_B), and FIC_B are defined in the same fashion for drug B.

for inherent variability in the technique and because a FIC index of 2.0 is probably indicative of an indifferent, rather than a true antagonistic, effect (13). In some cases it may be appropriate to use a more rigorous definition of synergism (e.g., a FIC index of less than 0.5) to discriminate more completely between synergistic and additive effects (325, 481). Although most investigators utilize the FIC index calculation only for two-drug combinations, the same mathematics can be applied to combinations of three or more drugs (as shown by Berenbaum et al. [44]).

Limitations of the Technique

As practiced by most laboratories, the checkerboard method has several significant flaws. First, unless each of the tubes (or wells) without obvious macroscopic evidence of growth is sampled to determine microbicidal activity, it yields only inhibitory data. (Obviously, such sampling complicates this relatively simple test and is possible only for broth dilution studies. It is not possible to sample agar dilution studies for microbicidal data, except in a very qualitative fashion.) This lack of microbicidal data is a serious limitation, because the organisms most frequently submitted for such testing are from patients with infections that most clinicians believe should be treated with microbicidal therapy (e.g., endocarditis, meningitis, or osteomyelitis).

Second, both the FIC index calculation and the usual interpretation of the isobologram assume incorrectly that all antimicrobials have linear dosage-response curves (see "Kinetic Spectrophotometric Methods"). Although checkerboard results are often used to characterize the dose-response relationship between an antimicrobial and a microorganism, the checkerboard method as usually performed provides only all-or-none responses (i.e., growth or no growth) and is thus incapable of measuring the graded responses necessary to define dose-response curves.

Finally, because the results are usually examined only at one point in time, the checkerboard method typically provides a static, rather than a dynamic, view of antimicrobial interaction. Despite these limitations, the checkerboard technique is simple to perform and remains a widely used technique to assess antimicrobial combinations.

Killing Curves (Time-Kill Curves or Time-Kill Plots)

In contrast to the checkerboard technique, which typically provides only inhibitory data, the killing-curve technique measures the microbicidal activity of the combination being tested. For this reason, it is presumably

more relevant for clinical situations in which bactericidal therapy is desirable. The other major advantage of killing curves over the checkerboard technique is that they provide a dynamic picture of antimicrobial action and interaction over time (based on serial colony counts), as opposed to the checkerboard technique, which is usually examined only once (after 16 to 24 hours of incubation).

However, the repetitive colony counts that this technique requires are tedious and seriously limit the number of antimicrobial concentrations and combinations that can be tested with any one isolate. Therefore, if the laboratory results are to be useful in guiding therapy, it is essential that the antimicrobial concentrations tested be chosen carefully and that they represent concentrations that are achievable at the presumed site of infection. Because the medium is sampled repetitively for colony counts, killing-curve studies must be performed in liquid media.

Most experiments are performed with a final inoculum of 10^5 to 10^7 CFU/mL, which is produced by diluting an overnight culture of the pathogen in Mueller-Hinton broth or another suitable broth. It is usually convenient first to adjust the overnight culture (or logarithmic-phase culture if desired) to match the 0.5 McFarland (barium sulfate) standard (386) and then to dilute a second time with fresh broth and the appropriate amounts of antimicrobials in the tube or flask in which the study is to be carried out.

The initial sampling for colony counts should take place as soon as the inoculum is added (within 5 minutes). Assuming that the volume of the culture is at least 10 mL, it is usually convenient to take 0.5-mL samples for colony counts, which may be performed by one of several methods. Especially if short sampling intervals are being used, the culture flasks should be returned to the incubator immediately after the aliquots have been removed. At each time point, the flasks are sampled again and serial dilutions (in sterile saline or molten agar) are performed for colony counts.

Saline Dilution

As usually performed, this method uses a series of tubes containing 4.5 mL of sterile saline (0.9% NaCl) or another appropriate sterile diluent. One pipettes a 0.5-mL sample from the culture into the first tube (producing a 10^{-1} dilution) and then, after thorough mixing, transfers 0.5 mL from that tube (with a new sterile pipette) to the next tube (which becomes the 10^{-2} dilution), repeating the procedure until a series of tubes have been prepared (usually from 10^{-1} to 10^{-8}). Precise aliquots from these tubes are then plated on an agar plate without antimicrobials and incubated overnight (or longer, for slowly growing organisms). If aliquots of 0.1 mL are used, they should be spread on the surface of a whole agar plate. However, if smaller volumes are used (e.g., droppers with a volume of 0.025 mL), it is often feasible to use a single plate for the results of all dilutions from one colony count (Fig. 9.5). In either case, it should be possible to use a single sterile pipette (or dropper) to pipette all dilutions for a single colony count, by beginning with the most diluted tubes (e.g., working from the 10^{-8} dilution to the 10^0 dilution) after the dilutions have been performed. Replicate colony counts obtained in this fashion should agree within ±10%. In contrast, significant differences among antimicrobials and antimicrobial combinations are usually defined as 10- to 100-fold changes in the number of CFU per milliliter after 4 to 24 hours of incubation. If one expects that the colony count will be close to zero, it is helpful to place an aliquot of the undiluted sample in the center of the plate (with the micropipette method) or to use an extra agar plate with 0.1 mL of undiluted material (with the macro 0.1-mL pipette method). Obviously, one cannot use the same pipette to dilute repetitively beginning with the initial culture material without the potential for significant carryover of microorganisms.

In many cases, it is also necessary to ascertain that carryover of antimicrobials from the dilution tubes is not of sufficient magnitude to lower falsely the resulting colony counts. This is usually not a problem when counting is performed on drops derived from the fourth or fifth dilution tube or beyond, because the process of dilution usually reduces residual antimicrobial concentrations to negligible levels. It is important to note, however, that, for antibiotics demonstrating marked inoculum effects against the test microorganism, drug concentrations significantly below the MIC (derived at standard inocula) may inhibit the growth of the few colonies present in that given dilution. When there is doubt about the possible effects of antibiotic carryover, control experiments should be carried out in which small inocula of the test strain are suspended in saline, with or without antimicrobial, and then plated immediately on drug-free agar. Re-

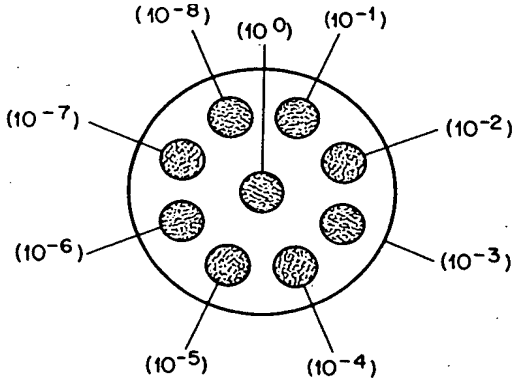


Figure 9.5. Arrangement of drops of culture broth on an agar plate for counting CFU per milliliter. *Shading*, area of the plate typically covered by single 0.025-mL drops from each 10-fold dilution.

sulting colony counts should be in close agreement in the absence of significant carryover effects.

Another approach is to inactivate the antibiotic(s) prior to plating, if this can be accomplished without affecting the organism. This is generally feasible only for β-lactam antibiotics, which can be inactivated by addition of commercially available β-lactamase. When significant carryover of a potent antimicrobial is anticipated, it may be necessary to collect bacteria by filtering aliquots through a 0.45-μm filter (e.g., Millipore Corp., Bedford, MA), washing the filter with sterile saline, and finally eluting organisms from the filter by vigorous agitation in a volume of saline equal to that of the original aliquot. The resulting bacterial suspension is then plated for colony counts. However, this process is tedious and requires that all specimens (including an antibiotic-free growth control) be handled in an identical fashion because of the inevitable retention of some colonies on the filter, even after vigorous agitation.

Another pitfall, which is somewhat less obvious, is the handling of the tubes with 4.5 mL of sterile saline. If these tubes are autoclaved after (rather than before) the saline has been added, the autoclaving process inevitably produces enough evaporation after the tubes are removed from the autoclave to significantly reduce the volume (usually to ≤4.0 mL). The only certain way to control this source of error is to dispense the sterile saline into the tubes after it and they have been sterilized.

The agar plates used for the colony counts must not contain any antimicrobials. Although Mueller-Hinton agar is quite sufficient for most bacteria, we have used blood, brain-heart, and nutrient agars for work with enterococci, and other media (e.g., chocolate agar) would clearly be necessary if one were to work with more fastidious organisms such as *Neisseria*. It is important not to tilt (and preferably not to move) the plates onto which drops have been applied until the drops have had time to dry thoroughly. This is less of a problem with 0.1-mL aliquots spread on individual agar plates, because the relatively small volume (in relation to the surface area) dries much more rapidly.

Agar Dilution

Colony counts may also be determined by agar dilution. Typically, a 1.0-mL aliquot is removed from the culture and diluted in sterile saline. Aliquots of this suspension are added to 19 mL of agar that has been allowed to cool to 50°C to 55°C after autoclaving, and the mixture is poured into agar plates (100 mm in diameter), allowed to harden, and then incubated overnight at 35°C to 37°C prior to colony counting. Penicillinase and sodium polyanetholsulfonate can readily be used to inactivate penicillin and aminoglycosides with the agar dilution method because it requires the pouring of fresh agar plates. In contrast, these agents (penicillinase and sodium polyanetholsulfonate) are used less frequently with the saline dilution method, because it does not involve the pouring of fresh agar plates. If penicillinase is added, 1.0 to 1.3 mL of penicillinase/20-mL plate (BBL, Cockeysville, MD) (the equivalent of 10^6 kinetic units of penicillinase/mL) is sufficient to inactivate several hundred micrograms of penicillin per milliliter in the sample. Sodium polyanetholsulfonate concentrations of 0.025 to 0.050 g/100 mL (or 5% NaCl) should inactivate clinically relevant concentrations of aminoglycosides and polymyxin (287).

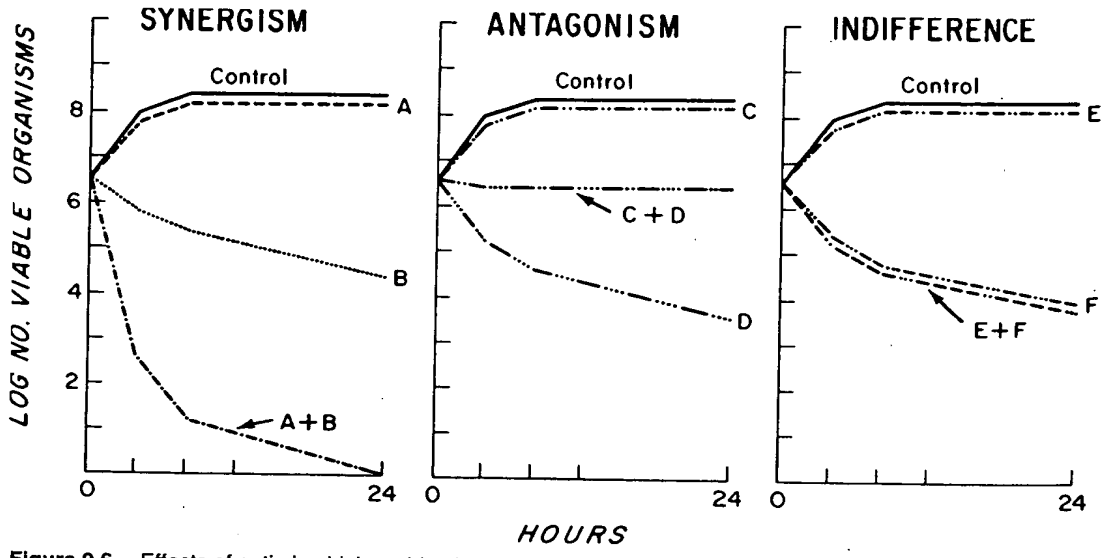


Figure 9.6. Effects of antimicrobial combinations as measured with the killing-curve method. *A + B*, synergism; *C + D*, antagonism; *E + F*, autonomy (or indifference).

After incubation of the agar plates at 35°C to 37°C for 16 to 18 hours (or less with some rapidly growing organisms), colony counts may be performed. When using the dropper technique, a colony counter with a magnifying glass is often helpful because up to several hundred colonies may be clustered in the approximately 1.5-cm-diameter circles produced by the drops on the agar plate (Fig. 9.5). When using the saline tube dilution method, we customarily place drops from each dilution tube onto duplicate antibiotic-free plates for counting and then average the results. Although there are usually fewer colonies per unit surface area with the macro technique (in which 0.1-mL aliquots of culture or diluted specimen are spread over the surface of a 100-mm agar plate), colony-counting devices are also helpful with that method.

Interpretation of the Results

When the colony counts have been determined, the easiest way to visualize the results is to plot them on semilogarithmic paper (using the abscissa for time and the ordinate [the logarithmic scale] for the colony counts, as in Fig. 9.6). If arithmetic paper is used, colony counts between powers of 10 should be plotted (on the ordinate) as their logarithms, rather than arithmetically, i.e., 3.1×10^7 (not 5×10^7) CFU/mL should be midway between 10^7 and 10^8 CFU/mL. The definitions of antimicrobial interaction with this technique are based on studies with enterococci, against which clinically acceptable concentrations of the aminoglycosides alone are generally inactive and penicillin is only bacteriostatic.

The results are interpreted by the effect of the combination in comparison with the most active single drug alone. Synergism is defined as a ≥100-fold increase in killing at 24 hours (as measured by colony counts) with the combination, in comparison with the most active single drug. Antagonism is defined as a ≥100-fold decrease in killing at 24 hours with the combination, compared with the most active single drug alone (366, 367). Additivity (or indifference) is defined as a less than 10-fold change (increase or decrease) in killing at 24 hours with the combination, in comparison with the most active single antimicrobial alone.

These definitions assume that at least one of the drugs being tested produces no significant inhibition or killing alone. Although this assumption is true for enterococci (against which clinically acceptable concentrations of aminoglycosides are inactive) (362), it is not true for most other organisms (especially Gram-negative bacilli), against which clinically acceptable concentrations of aminoglycosides are clearly active. At present, there are no established criteria with which to evaluate the results obtained (with the killing-curve technique) using two or more drugs, each of which has significant activity alone, to determine whether those drugs are synergistic. One approach has been to examine antimicrobials alone and then in combination at some fraction of the MIC or MBC (e.g., one fourth of the MIC or MBC). Typically, however, this results in some degree of killing with one or both of the agents used alone at an early time point, followed by regrowth at 24 hours. In such cases, the combination may result in sustained bactericidal activity over the entire sampling period, but it is difficult, if not impossible, to differentiate a true synergistic interaction from merely additive effects or from the phenomenon of mutual suppression of resistance to the single agents. Especially when penicillins are tested by time-kill methods against Gram-negative bacilli, late regrowth of microorganisms is often associated with loss of antimicrobial activity in the culture medium over time, due to slow inactivation of drugs. In such cases, determination of bactericidal activity of the single agents and combinations at earlier time points (e.g., 6 to 8 hours) may be more relevant (191).

Some authors have used statistical techniques to evaluate bactericidal interactions measured by killing curves

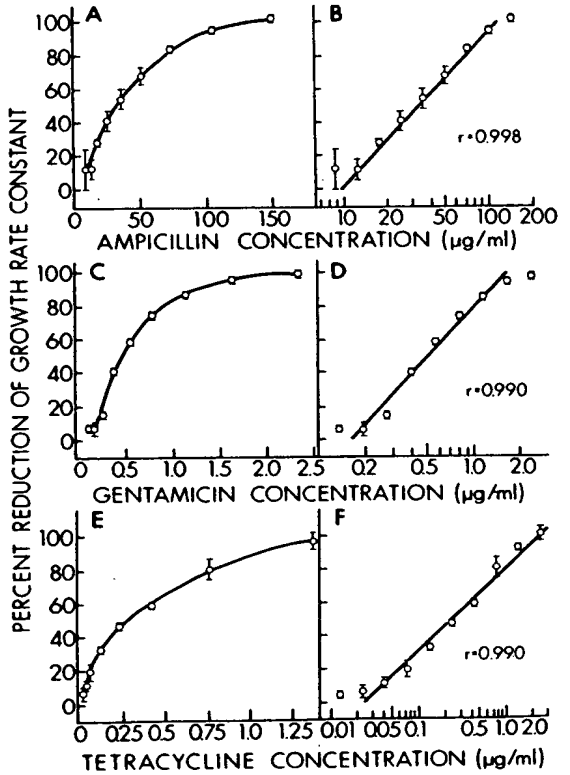


Figure 9.7. Effects of single antimicrobials on the bacterial growth rate constant. In each case, logarithmic plots of drug concentration on the abscissa (*right*) demonstrate a linear dose-effect relationship that is not apparent using an arithmetic scale (*left*), with *E. coli* American Type Culture Collection strain 25922. The individual data points represent the average of at least three determinations and are bracketed by the standard error of the mean. Correlation coefficients were calculated by the least-squares method for linear regression. (Reproduced from King and Krogstad [265], with permission.)

(209). In this method, survival probabilities at various time points for each drug and their combinations are examined for evidence of statistically significant differences.

Limitations of the Technique

The major disadvantage of the killing-curve technique is that the repetitive sampling necessary for each of the flasks being tested and the multiple colony counts required seriously limit the number of antimicrobial combinations that can be tested. For this reason, it is essential that the concentrations that are tested be chosen with knowledge of the antimicrobial concentrations that are achievable at the site(s) of the infection. Although this technique has been invaluable for understanding the response of serious enterococcal infections, the lack of a method to evaluate combinations in which each drug alone has significant antimicrobial activity seriously limits its usefulness in evaluating the activity of antimicrobial combinations against Gram-negative bacillary pathogens.

Serum Bactericidal Testing

Rationale

As noted above, one limitation of the killing-curve technique is that the few antimicrobial concentrations tested must represent concentrations achievable in vivo. To ensure that the antimicrobial concentrations tested are relevant clinically, several groups have employed the serum bactericidal titer as an estimate of the activity of antimicrobial combinations in patients receiving them (104, 130). The advantage of this approach is obviously that it measures the activity of antimicrobial concentrations that are achievable in vivo. Its disadvantages include the need to measure the levels of the different antimicrobials the patient is receiving in order to evaluate the results, the uncertainties introduced by the presence of antibody and complement in the serum specimen, and the added precautions needed to process patient serum specimens in the laboratory.

Interpretation of the Results

Because the amounts of antimicrobials in the serum specimen may vary greatly among patients on the same regimen, these titers are usually interpreted qualitatively. One practical approach is simply to repeat the test after the addition of an extra drug—to determine whether that drug enhances the activity of the initial agent (or combination). However, the use of serum bactericidal titers in this fashion does not exclude the possibility that the increment in activity observed results from the last agent added (alone), rather than from antimicrobial interaction. For this reason, the results obtained with this method should be described qualitatively and not as synergism or antagonism, unless several serum samples are also available that contain similar concentrations of each drug alone.

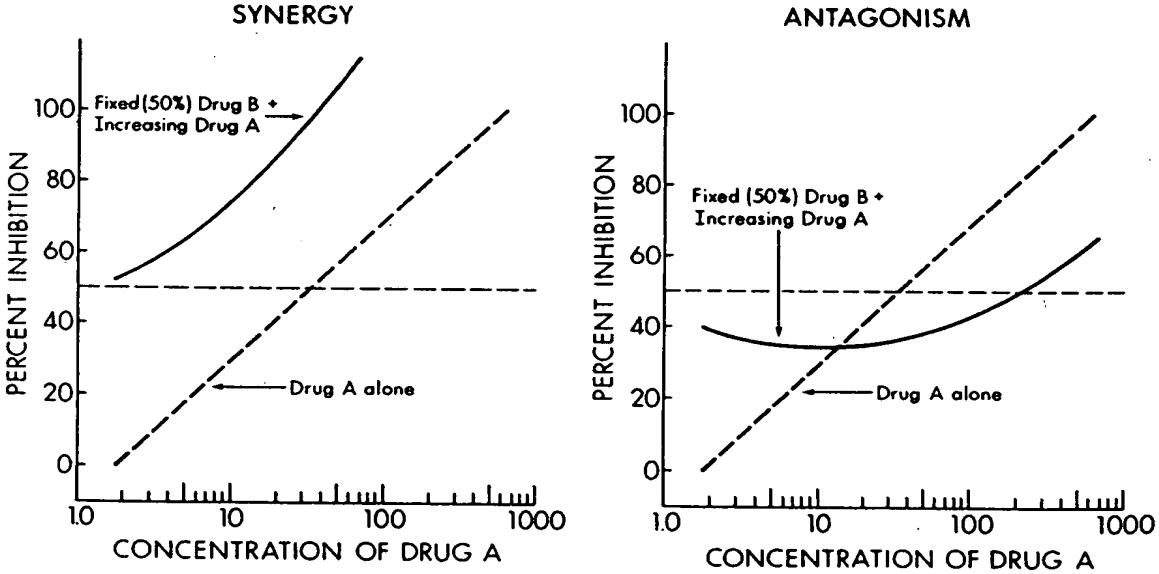


Figure 9.8. Effects of antimicrobial combinations on the growth constant as defined spectrophotometrically, using progressively increasing concentrations of one drug and a fixed concentration of the other calculated to produce a 50% reduction of the growth rate constant (50% inhibitory concentration). This approach maximizes the differences between autonomy and additivity and defines both synergism (a growth rate constant less than the additive growth rate constant) (*left*) and antagonism (a growth rate constant greater than that observed with either drug alone) (*right*). (Adapted from King and Krogstad [265], with permission.)

Kinetic Spectr phot metric Methods

Rationale

Spectrophotometric methods have been examined in the hope that they might provide both a more kinetic view of antimicrobial interaction and a more quantitative analysis of the dose-response relationship than is possible with the checkerboard technique (which is capable of generating only all-or-none, i.e., growth or no growth, responses unless the individual wells or tubes are sampled to determine killing). Because spectrophotometric methods are capable of measuring graded responses, one important result of these studies has been the demonstration that virtually all antimicrobial dose-response curves are logarithmic (i.e., exponential), rather than linear (Fig. 9.7). Another important theoretical result of these studies has been a reexamination of additivity and its biologic significance. Although additivity is interpreted (in the checkerboard system) as the result with antimicrobials that do not interact with one another, studies of auxotrophic mutants indicate that only one metabolic pathway is growth rate-limiting for an organism at a time. Thus, those studies suggest that, if two antimicrobials do not interact with one another, the result of those drugs (in combination) should be simply the effect of the more active drug alone, i.e., autonomy (266).

Method and Interpretation of the Results

Although spectrophotometric studies of bacterial growth have been performed for decades, the advent of sophisticated software has made it possible to monitor multiple cultures at frequent intervals while continuing to incubate those samples (265). With such equipment, one can plot growth curves for multiple cultures simultaneously and examine the effects of different antimicrobials and antimicrobial combinations on the growth-rate constants of those cultures. For experimental purposes, one can maximize the potential differences between autonomy and additivity in such a system by using the expected 50% inhibitory concentration (50% effective dose) of one drug (i.e., the concentration that reduces the growth-rate constant by approximately 50% when used alone) in combination with a steadily increasing series of concentrations of a second drug (Fig. 9.8). The results obtained with such a system (Fig. 9.9) clearly indicate that interactions corresponding to the synergistic, additive, autonomous, and antagonistic patterns postulated in Figure 9.8 do occur and that those results are similar, in this system, to those observed (with similar drugs) with the checkerboard method (Table 9.5).

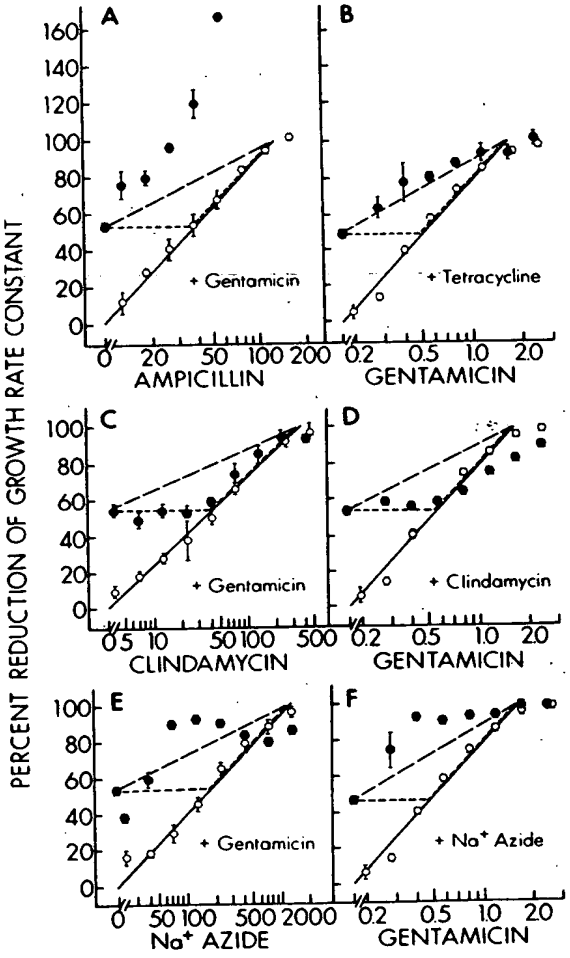


Figure 9.9. Kinetic spectrophotometric method. Using progressively increasing concentrations of one antimicrobial (designated on the abscissa) and a fixed concentration of the other (the 50% inhibitory concentration), the kinetic spectrophotometric method reveals synergism with ampicillin plus gentamicin (**A**), additivity with gentamicin plus tetracycline (**B**), autonomy with clindamycin plus gentamicin (**C**), autonomy and antagonism with gentamicin plus clindamycin (**D**), antagonism, synergism, and antagonism with sodium azide plus gentamicin (**E**), and synergism and autonomy with gentamicin plus sodium azide (**F**). ○, Single drugs; ●, combinations, with *E. coli* American Type Culture Collection test strain 25922. (Reproduced from King and Krogstad [265], with permission.)

Diffusion Method

A variety of methods have been explored to assess, primarily in a qualitative fashion, interactions of antimicrobials as they diffuse through agar plates seeded with a test organism. One major advantage of the diffusion method is that commercially produced antimicrobial-impregnated disks and Mueller-Hinton agar plates may be employed. Paper strips soaked in antimicrobial solutions are used in some modifications of this method. Therefore, it is not necessary to make up a series of flasks or agar plates containing different antimicrobial concentrations. The other major advantage of these techniques is (as described below) that they are easy to perform in the laboratory.

Table 9.5
Comparative Assessment of Antimicrobial Combinations by Spectrophotometric Measurement of Growth Rate Constants and by Checkerboard Testing[a]

Antimicrobial Combination	Method of Assessment	
	Spectrophotometric	Checkerboard
Ampicillin + gentamicin	Synergy	Additivity (1.0; 0.7–1.0)
Gentamicin + ampicillin	Additivity and synergy	
Tetracycline + gentamicin	Additivity	Additivity (0.9; 0.7–1.0)
Gentamicin + tetracycline	Additivity	
Clindamycin + gentamicin	Autonomy	Additivity (1.6; 1.0–1.7)
Gentamicin + clindamycin	Autonomy and antagonism	
Sodium azide + gentamicin	Antagonism, synergy, and antagonism	Antagonism (2.0; 1.0–2.0)
Gentamicin + sodium azide	Synergy and autonomy	
Chloramphenicol + clindamycin	Antagonism and autonomy	Additivity (1.6; 1.0–1.7)
Clindamycin + sodium azide	Additivity	Antagonism (2.0; 1.0–2.35

Reproduced from T.C. King and D.J. Krogstad (Ref. 265) with permission of *The Journal of Infectious Diseases* and the University of Chicag Press. © 1983, The University of Chicago.
[a]In the spectrophotometric experiments, the concentration of the first drug was progressively increased (using logarithmic increments o drug concentration), while the concentration of second drug was held constant at a concentration that reduced growth rate constant b approximately 50%. This strategy was chosen because it maximized the potential differences between autonomy and additivity. In th checkerboard experiments, the fractional inhibitory concentration (FIC) indexes are given as the result obtained with "equipotent" concen trations of both drugs (based on their MICs), followed by the range of FIC indexes observed with all concentrations tested.

Disk Technique

This technique uses the same standard inoculum and Mueller-Hinton agar as a routine Bauer-Kirby susceptibility test (37). To assess possible interactions between two drugs (e.g., drugs A and B), disks containing drugs A and B are placed on a plate that has been inoculated in the usual fashion with the organisms to be tested. The distance by which the disks are separated may be varied, but it should generally be equal to or slightly greater than the sum of the radii of the zones of inhibition of the drugs when examined alone. After overnight (16- to 18-hour) incubation at 35°C to 37°C, the plates are ready for examination.

The pattern observed with additive or indifferent combinations is that of two independent circles (Fig. 9.10*A*). With synergistic combinations, enhancement or bridging is observed at or near the junction of the two zones of inhibition (Fig. 9.10*B*). With antagonistic combinations, truncation is observed near the junction of the two zones of inhibition (Fig. 9.10*C*).

A special instance in which this technique can be very persuasive is when neither drug A nor drug B alone inhibits the test organism. In this situation, inhibition of growth can only be due to the combined effects of drugs A and B (Fig. 9.10*D*) (360). An example of an actual experiment showing synergism and antagonism is shown in Figure 9.11.

Another modification of this technique is the comparison of zones of inhibition produced by drugs A and B alone with the zone produced by a single disk containing both A and B. Although a larger zone of inhibition around A plus B than around either drug alone excludes significant antagonism, it may be impossible to distinguish between additive and synergistic interactions unless disks containing two times A and two times B are also tested. (The simplest instance is that found when zones of inhibition for A and B are equal, in which case the zone of inhibition for A plus B must be significantly greater than that of disks containing two times A or two times B to satisfy the criteria for synergism.)

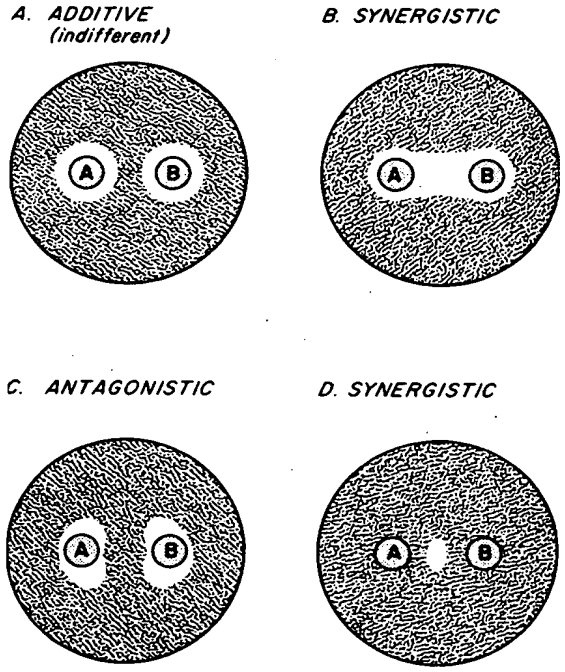


Figure 9.10. Assessment of antimicrobial combinations with the disk diffusion technique, using disks containing only one antimicrobial. **A.** Additive or autonomous result. **B** and **D.** Synergism. **C.** Antagonism. *Shading*, bacterial growth; *clear areas*, zones of growth inhibition.

Although the advantages of this technique as described are its simplicity and the use of readily available

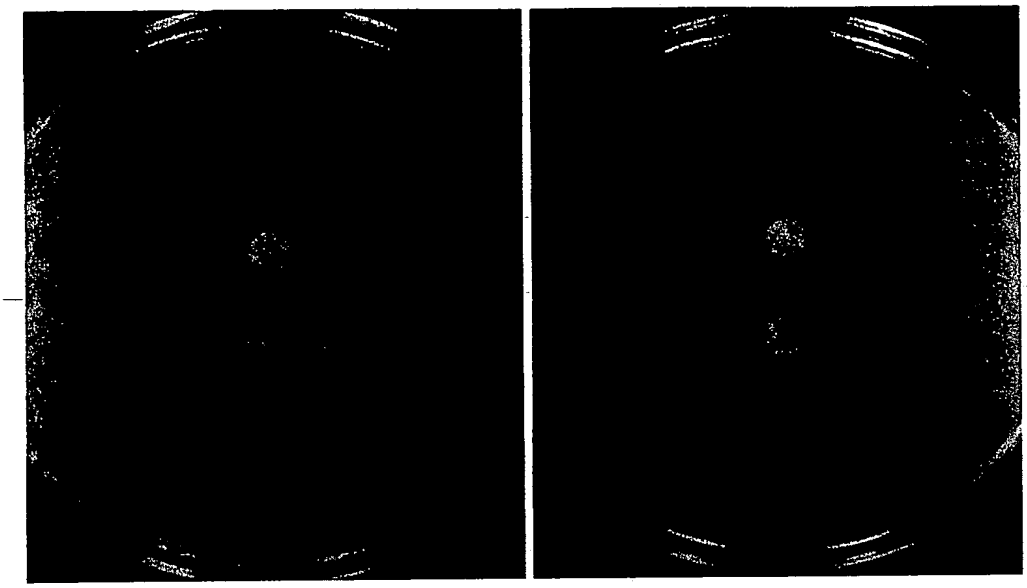


Figure 9.11. Assessment of antimicrobial combinations with the disk approximation technique. Combinations of β-lactams were tested against one strain of *E. cloacae*. *Left*, synergism between the two drugs. *Right*, activity of one drug is antagonized by the second, at concentrations below those that inhibit growth of the organism.

materials, it yields only qualitative information about antimicrobial interactions. Moreover, the results of this test may differ from results obtained when the same agents and organisms are tested in liquid media. As noted, it may be difficult to distinguish additive from synergistic interactions using this technique. Information about bacterial killing may be obtained by adding an agent to inactivate one or both of the drugs employed, thus permitting the detection of organisms that have been inhibited but not killed (e.g., the addition of β-lactamase to a penicillin-containing plate) (299, 589). A less specific method that provides similar information is the use of a velvet impression from the test plate, which may then be replica-plated onto an agar plate without antimicrobials (298).

Paper Strip Diffusion with Cellophane or Membrane Filter Transfer

In this method, which has been described by a number of investigators (51, 85–87, 134, 318), filter paper strips (0.5 to 0.9 × 4 to 5 cm) are soaked in antimicrobial solution and placed at right angles to one another on an agar plate (Fig. 9.12). After overnight incubation at 35°C to 37°C, the filter paper strips are removed, leaving behind drug that has diffused into the agar medium. After removal of the filter paper strips, a transferable material (cellophane [86] or a filter membrane [318]) that permits diffusion of the two drugs now contained on the agar plate is placed on the agar surface and inoculated with a suspension of the organism to be tested. After 16 to 18

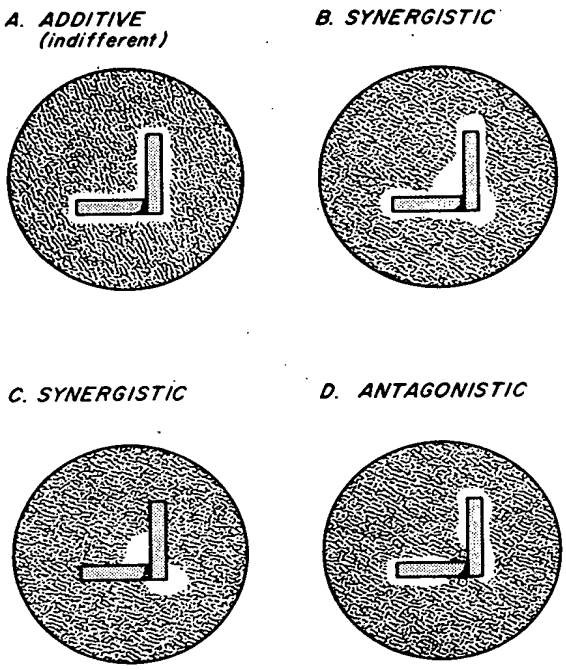


Figure 9.12. Assessment of antimicrobial combinations with the paper strip diffusion technique, using paper strips containing only one antimicrobial. **A.** Additive or indifferent result. **B** and **C.** Synergistic results. **D.** Antagonistic result. *Shading* and *clear areas*, as in Figure 9.10. See text for details.

hours of incubation, the transferable material is removed from the agar plate containing drugs A and B and transferred to another agar plate without antimicrobials. After an additional 18 hours of incubation at 35°C to 37°C, the growth pattern is examined.

The filter paper test may be modified by streaking culture plates with enough organisms to produce confluent growth. Filter paper strips soaked in different antimicrobials (in concentrations equal to or greater than the MIC for the organisms to be tested) are then placed on the plate at right angles. After several hours at room temperature (to allow the antibiotics to diffuse into the agar), the filter paper strips are removed and the plates are incubated for 18 to 24 hours at 35°C to 37°C. The patterns of growth inhibition that result with both methods are interpreted as described below (Fig. 9.12) (500).

Additive or indifferent combinations show no enhancement of their zones of inhibition at the former junction of the filter papers (Fig. 9.12*A*). Synergistic combinations show enhanced inhibition (Fig. 9.12*B*) and may rarely demonstrate activity at this point, even when the organism is resistant to both drugs separately (Fig. 9.12*C*) (318). Antagonistic combinations reveal truncated zones of inhibition at the former junction of the paper strips (Fig. 9.12*D*).

Advantages of this technique include its simplicity and the absence of equivocal results in the hands of experienced investigators (318). Disadvantages include the fact that it measures inhibition of bacterial growth rather than bactericidal activity, it is qualitative, and it is difficult to relate to drug concentrations that are achievable in humans. Although the absence of growth on drug-free media suggests a bactericidal effect, drug carryover has been shown to occur with the cellophane paper transfer technique (87). Therefore, unless drug carryover has been excluded, it is most reasonable to regard this test as an assay for growth inhibition or bacteriostasis, rather than for bactericidal activity.

Other Methods of Assessing Antibacterial Combinations

Some workers have used regression lines derived from least-squares analysis of MIC (x axis) versus zone diameter (y axis) to quantitate the effectiveness of combination therapy in vitro (128). They have suggested that synergism is indicated by a steeper negative slope (i.e., a more negative value of m in the equation $y = mx + b$) of the regression line for the combination of the two drugs, compared with each drug alone. Because the data on which each line has been based span only a short distance on the abscissa, their validity outside this range remains unclear. In addition, multiple MIC and zone size determinations with different organisms are required to determine a single line. Therefore, this method is of limited value when examining a single isolate for the presence of synergism with a given drug combination. When consecutive rather than simultaneous exposure of bacteria to two or more antibiotics is desired, the membrane transfer method may be used.

Sanders et al. (481) have described a technique utilizing graphic and statistical analysis of inhibition zone sizes using disks impregnated with various concentrations of each study drug. Concentrations are selected to represent decimal fractions of a "biological equivalence factor" equivalent to the amount of each agent producing identical zone sizes. Results from the combinations are compared with expected additive effects (each drug combined with itself). This method ("decimal assay for additivity") requires preliminary steps to establish biological equivalence factors and appropriate zone size ranges. However, it may be useful for detailed study of interactions for research purposes.

Mathematical Models to Assess Interactions

Various authors have applied mathematical methods in an effort to enhance the validity of conclusions regarding drug interactions when dose-response relationships are nonlinear or to display concentration-effect relationships three-dimensionally. Li et al. (309) described a method utilizing initial bactericidal rate constants at various drug concentrations. Pharmacodynamic models are tested to describe appropriate concentration-response curves, and results are expressed with isobolograms on two-dimensional plots where axes represent fractional maximal effects for each drug (on a scale of zero to one). A method of exploring drug interactions derived from nonlinear enzyme kinetic equations described by Chou and Talalay (95) has recently been applied in studies of antiviral drugs against human immunodeficiency virus (103, 280). In these examples, measurements of p24 antigen production, reverse transcriptase activity, etc., are taken at various concentrations of the drugs alone and combined.

Both activities and toxicities of antiviral combinations have been examined using a three-dimensional graphic method described by Pritchard and Shipman (121, 441, 442). Concentrations of the two drugs tested are plotted along x and y axes forming a horizontal plane, and the effect being measured (e.g., p24 antigen) is plotted on the z axis perpendicular to the plane. Plotting of experimental data points (with the aid of a microcomputer) yields a three-dimensional surface. A second surface representing the "additive" interactions generated from dose-response curves of individual agents is subtracted from this, leaving a series of peaks and pits corresponding to areas of significant deviation from additivity (442). Assumptions inherent to each of these models have been discussed elsewhere (309, 442).

Summary of Methods for the Study of Synergism

The salient features, advantages, and disadvantages of several methods have been tabulated (Table 9.6). Before selecting a specific technique, it is useful to consider

Table 9.6
Comparison of Different Techniques Used to Assess Antimicrobial Combinations

Technique	Medium	Antibacterial Effect	Modifications	Advantages/Disadvantages
Checkerboard	Broth dilution	Bacteriostatic	Killing can be assessed by sampling of clear tubes/wells	Many different combinations and concentrations can be tested, but bactericidal data can be extracted only with difficulty; does not assess rate of killing
	Microdilution in broth	Bacteriostatic		
	Agar dilution	Bacteriostatic	Microdilution on agar	
Killing curve	Broth	Bactericidal		Few combinations can be tested but the information produced is relevant to bacterial killing over time at defined drug concentrations
Disk diffusion	Agar	Bacteriostatic	–	Diffusion of antimicrobials produces a gradient of drug concentration without a clear relationship to clinically achievable drug levels; bactericidal data are difficult to obtain
Paper strip diffusion	Agar	Bacteriostatic	–	
Kinetic Spectrophotometric	Broth	Bacteriostatic	Killing can be assessed by performing colony counts at multiple points during the experiment	Provides a potentially helpful kinetic view of antimicrobial action and interaction; bactericidal data can be obtained only by colony counts

both the laboratory's capabilities and the type of information that would potentially be most helpful. For situations in which simplicity is important, diffusion disk testing and filter paper strips with or without transfer techniques are probably reasonable choices. However, it must be noted that these methods are strictly qualitative and have not been widely validated by comparison with more quantitative methods. If many different combinations of the drugs must be examined, a checkerboard may be optimal. If the MIC and MBC are close to one another, the result of checkerboard testing using agar and broth dilution techniques may be in good agreement with killing-curve data. However, if the MIC and MBC are disparate and bactericidal therapy is crucial, a killing curve is the most appropriate test. Other problems in utilizing these tests are basically those encountered in routine broth dilution and agar diffusion susceptibility testing.

Inoculum

For clinical purposes, four or five colonies of the same morphology should be used to inoculate broth cultures (560). For research purposes, single-colony isolates that have been purified (streaked to single colonies) at least twice should be employed. Clearly, a mixed culture cannot produce a satisfactory result.

Standardization of the Inoculum

As noted above, an excessively heavy inoculum tends to produce falsely high MIC and MBC values with all methods described, and an excessively light inoculum tends to produce a falsely low value. This tendency is typically accentuated with organisms capable of inactivating the antimicrobials being tested (e.g., in the testing of β-lactamase-producing organisms with penicillin). When determining the MBC it is important to use a large enough inoculum (3 to 10 × 10^5 CFU/mL) so that ≥99.9% killing can be measured accurately (424).

Media

Mueller-Hinton agar and broth are recommended for susceptibility testing because they permit growth of the most common pathogens and contain few substances that interfere with antimicrobial activity. Exceptions to this generalization include the use of specialized media for fastidious organisms and the study of antimicrobials subject to antagonism by certain medium components, e.g., chocolate agar for *Neisseria* spp. and the addition of lysed horse blood for TMP/SMZ testing (220) (see below).

Because the action of several antimicrobials is pH dependent, another cause of difficulty is failure to adjust the pH of the medium to between 7.2 and 7.4. pH levels below 7.0 begin to inactivate most aminoglycosides (30, 335, 574). In addition to pH, aminoglycosides, fluoroquinolones, and polymyxins, as well as daptomycin, are sensitive to variations in the concentrations of Mg^{2+} and/or Ca^{2+} (113, 141, 147, 185, 449). Current recommendations call for media to contain 10 to 12.5 mg/liter Mg^{2+} and 20 to 25 mg/liter Ca^{2+} (386).

ANTIMICROBIAL INTERACTIONS RESULTING IN ANTAGONISM

From a clinical viewpoint, antagonism is the most disadvantageous outcome possible with an antimicrobial combination, because the effect of the combination may be less than that of either drug alone. However, there are only a few reports in which clinically significant antag-

onism has been documented (304, 408, 569), despite the large number of reports of in vitro antagonism in the literature. It is possible that clinically significant antagonism is often not recognized in patients with complex disease states or that investigators who suspect it may be reluctant to report adverse results. It is also possible that the antagonisms defined under relatively controlled conditions in vitro may not be significant in vivo, especially in the presence of intact host defenses. For whatever reason, documented reports of clinically significant antagonism are uncommon.

Combinations of Bacteriostatic Agents with β-Lactam Antibiotics

The clearest example of clinically significant antagonism was reported in a study of pneumococcal meningitis (304). In that study, patients who were treated with penicillin/chlortetracycline had a mortality rate of 79%, versus a mortality rate of only 21% for patients who were treated with penicillin alone. Those results presumably reflected antimicrobial antagonism in vivo, because tetracycline also prevents the bactericidal action of penicillin against the pneumococcus in vitro. Although the exact mechanism responsible for this effect is unknown, it is known that a bacterium must be growing for penicillin to express its bactericidal activity and that the bacteriostatic action of tetracycline on the ribosome inhibits bacterial growth. An additional potential mechanism is inhibition (by tetracycline) of the production of autolysin by the pneumococcus (533).

Like tetracycline, chloramphenicol (a reversible inhibitor of protein synthesis that binds to the 50S subunit of the ribosome) has also been shown to antagonize bacterial killing by penicillin in vitro (248, 443) and to reduce the activity of newer β-lactams such as cefotaxime, moxalactam, cefoperazone, and aztreonam against *Klebsiella pneumoniae* (58). Although such studies raise serious questions about the use of chloramphenicol/β-lactam combinations (especially for meningitis), attempts to reproduce this phenomenon in vivo have met with mixed success. For example, antagonism has been demonstrated in the dog model of pneumococcal meningitis only when chloramphenicol is administered before penicillin. When the drugs are given simultaneously or when penicillin is given first, antagonism is less marked or absent. Regardless of the initial protocol, continued administration of both drugs produced complete killing of the organism in this model (558).

More recent studies have failed to demonstrate antagonism with penicillin/chloramphenicol combinations in the rabbit model of meningitis with *Haemophilus influenzae* or *Streptococcus pneumoniae* (50). The fact that penicillin does not inhibit the antibacterial activity of chloramphenicol (even though chloramphenicol is less effective than penicillin against both pneumococci and *H. influenzae*) may account for the lack of significant in vivo antagonism in these studies. There have been no convincing reports of penicillin/chloramphenicol antagonism against these pathogens in humans.

Studies of the autolytic enzyme system indicate that chloramphenicol inhibits the activity of the autolysin responsible for the penicillin-induced lysis and killing of pneumococci (533). The fact that chloramphenicol also inhibits bacterial growth and protein synthesis may help to account for its ability to inhibit the bactericidal action of penicillin in vitro. Chloramphenicol also antagonizes the bactericidal effect of ciprofloxacin against *S. aureus*, *Escherichia coli*, and *P. aeruginosa* (447, 602). The exact interactions leading to this effect have not yet been established.

Combinations of 50S Subunit Ribosomal Inhibitors

Many antimicrobials (erythromycin, lincomycin, clindamycin, spiramycin, oleandomycin, sparsomycin, puromycin, and chloramphenicol) inhibit bacterial protein synthesis by binding to the 50S subunit of the ribosome. Because a number of these agents bind to the same (or very similar) sites on the ribosome (576), the use of more than one of these antimicrobials may result in competition for target sites, with resultant loss of activity of the most active compound. For example, lincomycin, erythromycin, oleandomycin, and spiramycin have been shown to inhibit the binding of chloramphenicol to intact *Bacillus megaterium* and to ribosomes isolated from them (554). In addition, *S. aureus* strains resistant to erythromycin also become resistant to lincomycin or spiramycin if they are exposed to combinations of erythromycin plus lincomycin or erythromycin plus spiramycin, respectively (29). Similar observations have been made with the combination of erythromycin plus clindamycin with *Staphylococcus epidermidis* (314). These effects most likely arise from induction of methylase enzymes by erythromycin, which renders the ribosomes resistant to the other macrolides/lincosamides (which are themselves less potent inducers of this system), rather than from direct competition between the drugs for ribosomal target sites (359).

It also appears that the physical state of the ribosome may be an important determinant of the activities of these drugs. For example, the macrolide antibiotics (erythromycin, oleandomycin, and spiramycin) prevent the binding of chloramphenicol to individual 70S ribosomes but not to polysomes (ribosomes on messenger RNA), thus raising serious questions about the validity of observations made with these agents in cell-free systems (429).

Although there is convincing evidence that antagonism can occur in vitro with these agents (particularly with two macrolide antimicrobials that both bind to the 50S subunit of the ribosome), there have been no clinical reports (of which we are aware) to establish that these observations are relevant in vivo. The lack of such reports is particularly striking because the combination of erythromycin plus chloramphenicol was used to provide

broad-spectrum coverage for many years before the advent of the newer aminoglycosides and cephalosporins (443).

Combinations of Aminoglycosides with Bacteriostatic Agents

A number of investigators have shown that the bactericidal activity of the aminoglycosidic aminocyclitols (such as gentamicin, tobramycin, amikacin, netilmicin, kanamycin, and streptomycin) is antagonized in vitro by bacteriostatic agents such as tetracycline or chloramphenicol (247, 443). Although the exact mechanism responsible for this antagonism remains unknown, it is possible that chloramphenicol and tetracycline inhibit the active transport mechanisms necessary for the energy-dependent uptake of aminoglycosides into bacterial cells (60) or that they prevent the movement of the ribosome along messenger RNA, so that it cannot return to form the initiation complex that appears to be the critical site for the bactericidal action of aminoglycosides (443).

Several studies have demonstrated in vivo antagonism of aminoglycoside action by chloramphenicol in experimental animal models. Combinations of gentamicin plus chloramphenicol are antagonistic in the treatment of meningitis due to *Proteus mirabilis* in rabbits (519) and for *P. mirabilis* peritonitis in neutropenic mice (472). Studies of *Serratia marcescens* peritonitis in normal mice have been less clear (443). Although there are no published reports (of which we are aware) of antagonism in humans with these combinations, the animal studies cited above suggest that aminoglycoside plus chloramphenicol (or tetracycline) combinations should be used with caution, if at all, in the treatment of immunosuppressed patients or in the treatment of meningitis.

Several reports have suggested that the combination of erythromycin (or clindamycin) plus gentamicin may be synergistic by the checkerboard method (162, 163, 303). However, time-kill curve studies with these same combinations suggest that clindamycin may antagonize the early bactericidal activity of gentamicin (610).

Inactivation of Aminoglycosides by β-Lactams

Prolonged exposure of aminoglycosides to β-lactams clearly results in inactivation of the aminoglycosides (331, 455, 556). If the two antimicrobials are mixed in the same intravenous bottle and allowed to stand, this inactivation may be great enough to cause the failure of the antimicrobial regimen. Because both β-lactams and aminoglycosides are normally excreted by the kidneys at a rate much greater than the rate of drug inactivation, this chemical interaction is normally insignificant unless the patient has serious renal compromise. Although amikacin is more resistant to this inactivation than are the other aminoglycosides in common use (192), the doses of all aminoglycosides must be carefully adjusted in these patients (by measuring serum levels), whether or not they are also receiving β-lactams. Because this phenomenon takes place in the absence of the microorganism, it falls outside the realm of antagonism as defined in this chapter and is not discussed further.

Combinations of β-Lactams

A number of troublesome Gram-negative pathogens, such as *P. aeruginosa*, *Enterobacter* spp., *Citrobacter*, and *Serratia*, possess chromosomally mediated β-lactamases that are inducible upon exposure of the organisms to β-lactam antibiotics (197). A number of β-lactamase-resistant cephalosporins not only are potent inducers of these enzymes but also may facilitate selection of stably derepressed (i.e., fully induced) mutant strains of these organisms (198, 474, 476–478). Against strains possessing such enzymes, the combination of a potent inducer of chromosomal β-lactamase (e.g., cefoxitin) with an intrinsically more potent agent that is not fully resistant to hydrolysis (e.g., a ureidopenicillin) may result in significant loss of activity of the latter. This is illustrated in Figure 9.11, where such a combination was employed against a strain of *Enterobacter cloacae*. Even drugs that appear to be relatively resistant to β-lactamase hydrolysis, such as the third-generation cephalosporins, suffer loss of activity against fully derepressed mutant strains.

Although alternative hypotheses have been proposed (474), this phenomenon is probably best explained by the combined roles of the permeability barrier created by the outer cell membrane of Gram-negative organisms and of the previously underestimated hydrolytic capacity of periplasmic β-lactamases. Vu and Nikaido (555) have demonstrated that, in the presence of intact outer cell envelope barriers to the penetration of β-lactams, the periplasmic β-lactamases of induced cells are capable of hydrolyzing the low concentrations of (even relatively "stable") cephalosporins or penicillins that can be achieved in this space.

Several animal models have been used to demonstrate that β-lactam/β-lactam antagonism also occurs in vivo (194, 289). Although the clinical significance of these observations was not clear initially (477, 567), several investigators have now shown that these same phenomena may occur clinically, resulting in bacteremias and other serious infections with organisms that have become resistant to the β-lactams initially used for their treatment (380, 476).

During treatment of *Enterobacter* spp. bacteremias with cephalosporins to which the organisms appear susceptible by standard laboratory tests, a substantial breakthrough rate of stably derepressed mutants has been documented (98). Sanders (475) has described such Gram-negative organisms with inducible chromosomal β-lactamases as "not truly susceptible to the newer-generation cephalosporins," because the resistance genes are present but not activated during routine susceptibility testing.

Combinations of Quinolones with Other Agents

Because of widespread interest in the clinical use of fluoroquinolone antimicrobials (e.g., ciprofloxacin and ofloxacin), several studies have explored combinations of these drugs with a variety of other antibiotics. Fortunately, such combinations have demonstrated antagonism relatively infrequently (140). In vitro, chloramphenicol antagonizes the bactericidal activity of ciprofloxacin against some strains of *S. aureus*, *E. coli*, and *P. aeruginosa* (447, 602). By time-kill methods, the bactericidal rate of ciprofloxacin against staphylococci can be reduced in the presence of other agents demonstrating even weaker bactericidal activity (e.g., vancomycin [418] or rifampin [549]). However, because such combinations do not inevitably result in decreased serum bactericidal activities (relative to the most active drug alone) and may result in the mutual suppression of resistant subpopulations, it is not certain that such potential antagonism would be clinically relevant. Antagonistic effects in vitro between fluoroquinolones and novobiocin, an older antibiotic that also inhibits DNA gyrase, have been reported against staphylococci (308).

ANTIMICROBIAL INTERACTIONS RESULTING IN SYNERGISM

Having introduced the most frequent justifications for the use of combination therapy and the techniques commonly used to assess antimicrobial interaction, we now consider the major mechanisms responsible for antimicrobial synergism against bacteria.

There are four generally accepted mechanisms of antibacterial synergism: (*a*) serial (sequential) inhibition of a common biochemical pathway (e.g., TMP/SMZ), (*b*) inhibition of protective bacterial enzymes (e.g., cloxacillin, clavulanic acid, or sulbactam plus a β-lactamase-susceptible penicillin), (*c*) use of combinations of cell wall-active agents (e.g., amdinocillin [mecillinam] plus ampicillin), and (*d*) use of cell wall-active agents to enhance the uptake of other antimicrobials (e.g., penicillin plus streptomycin). In addition, there are several instances in which the criteria for synergism are fulfilled but the mechanism of drug interaction is unclear (e.g., SMZ plus colistin).

In this section we consider each of these groups of interactions, beginning with the known mechanisms of action and resistance (citing examples from both laboratory and clinical studies) and concluding with a discussion of the laboratory results and their clinical application, as well as simple tests that may be used to predict resistance to combination therapy.

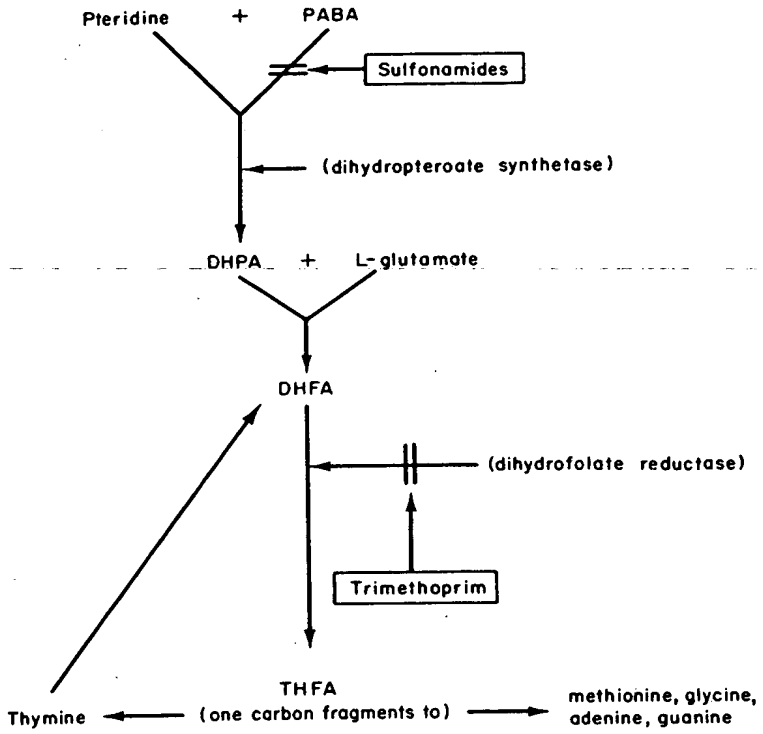
Sequential (Serial) Inhibition f a Comm n Biochemical Pathway

The best known example of antimicrobial synergism by sequential inhibition of a common biochemical pathway is the combination of TMP and SMZ. Two additional combinations that most likely also involve sequential inhibition of a common pathway are those containing amdinocillin (mecillinam) or vancomycin plus penicillins or cephalosporins (25, 127). For the purposes of this chapter, the latter interactions are considered as combinations of agents acting on the cell wall, although they probably act in sequence on biochemical pathways involved in cell wall synthesis.

Mechanism of Action

SMZ and other sulfonamides are thought to exert their antibacterial effect by competing with *p*-aminobenzoic acid to prevent the formation of 7,8-dihydropteroate (Fig. 9.13) (57). 7,8-Dihydropteroate is combined with L-glutamate to form dihydrofolic acid and is then reduced (in the presence of dihydrofolate reductase) to tetrahydrofolic acid, which is an essential donor of one-carbon fragments for the synthesis of thymidine, methionine, glycine, adenine, and guanine in both bacterial and mammalian cells (Fig. 9.13) (137, 225). TMP, pyrimethamine, and other antifolate agents inhibit dihydrofolate reductase and thus inhibit the production of tetrahydrofolic acid from dihydrofolic acid (Fig. 9.13) (226). Because the effects of TMP plus SMZ, including cell death, can be reversed by thymidine, thymineless death is thought to be the mechanism by which TMP/SMZ exerts its bactericidal effect (279, 531). Bacterial cells generally cannot absorb exogenous folates; thus, they are unable to bypass the block created by TMP plus SMZ (Fig. 9.13) (225). Mammalian cells, which can absorb exogenous folate, are also protected because they contain a different dihydrofolate reductase. TMP is more than 10,000 times more active against bacterial dihydrofolate reductase than it is against the mammalian enzyme (64, 226, 255).

Although the TMP/SMZ combination acts at at least two points in the folate pathway, there has been some uncertainty, on theoretical grounds, whether sequential inhibition of a linear pathway could produce a truly synergistic effect (568). Therefore, it has been proposed that sulfonamides may act synergistically with TMP on the same enzyme, dihydrofolate reductase (439). In support of this concept, it has been shown that a variety of sulfonamides synergize with TMP against an *E. coli* dihydrofolate reductase in vitro (439). However, the concentrations of sulfonamide used in those studies were several orders of magnitude greater than those achievable in vivo, and this mechanism would therefore require that the organism concentrate sulfonamide for synergism to occur either in vitro or in vivo (63, 530). Because there is currently no evidence for such high intracellular concentrations of sulfonamides, this seems unlikely. Other workers have pointed out that dihydrofolate reductase may be in a cyclic rather than a linear pathway (considering the recycling of tetrahydrofolate after it has been oxidized to dihydrofolate) (Fig. 9.13) and that sequential inhibition in such a system should therefore be capable



Figur 9.13. Mechanisms of action of sulfonamides and TMP on the folic acid pathway. *PABA*, *p*-aminobenzoic acid; *DHPA*, 7,8-dihydropteroate; *DHFA*, dihydrofolic acid; *THFA*, tetrahydrofolic acid.

of producing synergism if the sulfonamides reduce the quantity of intermediates available (63, 568). Another objection raised by some investigators against the aforementioned hypothesis was that several dihydrofolate reductase inhibitors with structures quite different from that of TMP were all capable of synergizing with sulfonamides, which would not have been expected if both compounds were acting together on the same enzyme (530).

In summary, although sulfonamides and TMP have been shown to synergistically inhibit dihydrofolate reductase activity in vitro, the concentrations of sulfonamide at which this occurs are significantly higher than those achievable in vivo, where synergism has been demonstrated (63, 68). At present, it seems reasonable to regard sequential inhibition of a common (probably cyclical) pathway as the most likely basis for the synergism observed with TMP plus SMZ.

Laboratory Studies of TMP plus SMZ

Laboratory studies have been performed on a wide variety of organisms, using many different methods. The drug concentrations most frequently employed have been a 1:20 ratio of TMP to SMZ (the usual ratio of serum concentrations achieved with oral administration of the commercially available 1:5 combination). This is because the optimal ratio of two drugs for the production of synergism is thought to be the ratio of their MICs for the organism being tested (68), and TMP is 20 to 100 times as active as SMZ against most bacteria. Therefore, using this fixed 1:20 ratio is usually appropriate. Nevertheless, this ratio may not be optimal for all organisms, because at least three genera (*Neisseria*, *Brucella*, and *Nocardia*) are more susceptible to SMZ than to TMP and should presumably be studied with a different TMP/SMZ mixture (68).

Gram-negative organisms often reported as susceptible to TMP plus SMZ in vitro include *H. influenzae* (329), *E. coli* (2), *Salmonella typhi* (72), *Shigella* spp. (460), *K. pneumoniae* (68), *Bordetella pertussis* (68), and *Enterobacter* spp. (68). Although many isolates are resistant, TMP/SMZ is one of the few antimicrobials with substantial activity against some strains of *Pseudomonas cepacia* and *Xanthomonas maltophilia* (307, 342). *P. aeruginosa*, which is generally resistant to both TMP and SMZ separately, is characteristically also resistant to TMP/SMZ synergism. Similarly, *Mycobacterium tuberculosis*, which is usually resistant to both drugs, has also been resistant to the TMP/SMZ combination (68).

In spite of their relative TMP resistance, a number of *Neisseria gonorrhoeae* strains are synergistically inhibited by TMP plus SMZ. This is most noticeable using a 3:1 combination (rather than the usual 1:20 combination) (22), which more closely approximates the ratios of the MICs of these two drugs for *N. gonorrhoeae*.

Among Gram-positive organisms, streptococci and pneumococci are susceptible to TMP plus SMZ, as are some strains of *S. aureus*. Enterococci (*Enterococcus*

faecalis) are typically resistant to TMP/SMZ synergism in vivo because they are able to absorb exogenous folate and thus bypass the metabolic block produced by the drug combination (Fig. 9.13) (68, 225, 226). Among the actinomycetes, synergism has been shown in vitro for *Nocardia asteroides* (332). Thus, the in vitro laboratory data suggest a wide range of bacteria against which the TMP/SMZ combination might be effective in vivo.

Mechanism of Resistance In Vitro

There are at least two mechanisms by which bacteria may be resistant to TMP/SMZ synergism in vitro (Fig. 9.14): (*a*) an altered dihydropteroate synthetase with a decreased affinity for sulfonamides and (*b*) an altered dihydrofolate reductase with a decreased affinity for TMP. Resistance to TMP based on intrinsically poor drug penetration (e.g., *P. aeruginosa*) or acquired reductions in bacterial cell envelope permeability (e.g., *K. pneumoniae*) has also been described (241).

Sulfonamide resistance was noted shortly after sulfa drugs were first introduced for clinical use. In 1952, it was postulated that the basis of this resistance might be an enzyme with decreased affinity for sulfonamides (117). Subsequently, in 1963, it was demonstrated that the dihydropteroate synthetase activity of sulfonamide-resistant *E. coli* mutants was resistant to sulfonamide inhibition (417). These findings were later corroborated by kinetic studies of dihydropteroate synthetase activity in sulfonamide-resistant *Neisseria meningitidis* and *N. gonorrhoeae* (229). It is now clear that the basis of this resistance in some clinical isolates of sulfonamide-resistant *E. coli* is a plasmid coding for a different dihydropteroate synthetase with decreased affinity for sulfonamides. Both the sulfonamide resistance phenotype and the less heat-stable enzyme (which has a lower molecular weight than the wild-type enzyme) were transferable to recipient strains (586). It was demonstrated in 1945 that some sulfonamide-resistant strains produced increased quantities of *p*-aminobenzoic acid (292). However, it is not certain that this was the cause of the resistance observed, and the significance of this observation in sulfonamide-resistant strains remains unclear, although it has subsequently been confirmed by other workers (243, 579).

TMP resistance was not a problem in early studies. TMP had been chosen from a group of several antifolates after examination of their activities against the dihydrofolate reductases of several species (64, 225, 226). The concentration of TMP necessary for 50% inhibition of human dihydrofolate reductase activity in these studies was 60,000 times that required for a similar effect on bacterial dihydrofolate reductase (*S. aureus*, *Proteus vulgaris*, and *E. coli*) (64, 225, 226). Thus, the choice of TMP as a therapeutic agent took advantage of naturally occurring species differences in enzyme function.

However, bacterial dihydrofolate reductases that are resistant to TMP have now been described (7, 422, 498). Transfer experiments in 1972 first suggested that this TMP resistance might be plasmid-mediated (170). Later

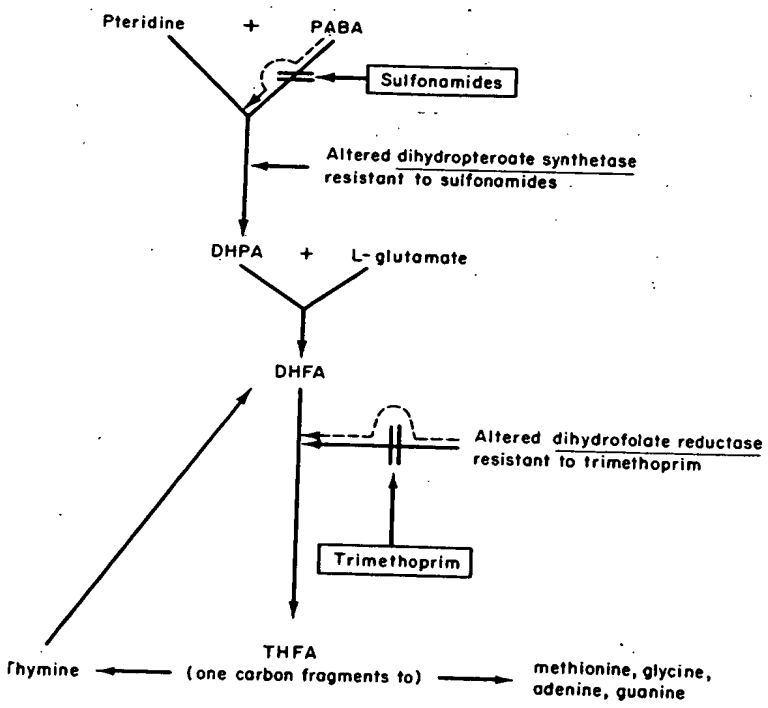


Figure 9.14. Mechanisms of resistance to sulfonamides and TMP in vitro. The most prevalent mechanisms of resistance are altered dihydropteroate synthetase and dihydrofolate reductase enzymes with decreased affinities for sulfonamides and TMP, respectively. See text for details; abbreviations are as in Figure 9.13.

work confirmed that the previously mentioned TMP-resistant dihydrofolate reductases are transferable (i.e., presumably plasmid-mediated) (7, 498). Two different plasmid-mediated, TMP-resistant dihydrofolate reductases, which could be distinguished by their relative susceptibility to TMP and by their pH optima (both had molecular weights of 32,000 to 37,000), were then described (422). Subsequent investigations determined the amino acid sequence of another TMP-resistant dihydrofolate reductase from *E. coli*, which is similar in molecular weight to the wild-type TMP-susceptible enzyme (517). Since that time, several additional distinct, plasmid-mediated, TMP-resistant dihydrofolate reductases have been recognized (241, 496).

Mechanisms of Resistance to TMP plus SMZ In Vivo

As mentioned above, enterococci are generally resistant to the TMP/SMZ combination in vivo because they are able to absorb the exogenous folates present in human plasma (44, 68, 225, 226). Two animal studies have demonstrated lack of activity of TMP plus SMZ against enterococcal infections in vivo, despite activity in vitro (93, 201). In the rat endocarditis model, numbers of residual bacteria in cardiac vegetations of TMP/SMZ-treated animals were identical to those of untreated controls (201).

Clinical Studies of TMP plus SMZ

Most clinical trials of TMP/SMZ synergism have used a fixed combination with five times as much SMZ as TMP (by weight). This combination usually produces serum levels of SMZ in humans approximately 20 times those of TMP (in micrograms per milliliter) (108, 161), which is close to the most frequent ratio of their MICs against common pathogens (see "Laboratory Studies of TMP plus SMZ").

Problems associated with the use of the TMP/SMZ combination include decreased intestinal absorption and inadequate serum antimicrobial concentrations in critically ill patients with ileus, and fluid overload with intravenous administration in patients with renal and/or cardiac failure (because both drugs are only sparingly soluble in water). TMP/SMZ toxicity is most frequently due to hypersensitivity reactions to one of the two components (especially SMZ). Although neutropenia and thrombocytopenia do occur with TMP plus SMZ, particularly in children (18), marrow depression secondary to the antifolate effect of TMP alone has not commonly been reported under ordinary circumstances (171). Higher frequencies of adverse effects, including hypersensitivity reactions and hematologic effects, however, have been observed in patients with human immunodeficiency virus infection (99). On theoretical grounds, it should always be possible to reverse marrow toxicity (when it occurs) with folinic acid, which mammalian cells (but not bacteria other than enterococci) can absorb from their external environment.

The primary route of excretion for both drugs is through the kidneys, with an elimination half-life of 11 to 14 hours (257, 400). Although the dose should therefore be reduced in patients with renal failure (577), TMP/SMZ has been successfully used for the treatment of urinary tract infections in these patients (40).

Substantial experience has now accumulated with the use of TMP plus SMZ for meningitis and brain abscess. The available data suggest that SMZ readily crosses the blood-brain barrier into the cerebrospinal fluid and that TMP penetrates well across mildly inflamed meninges. Levels of TMP and SMZ in brain tissue of a patient being treated for *N. asteroides* brain abscess were reported to be 5.1 and 36.0 μg/g of tissue, respectively, 6 hours after the last TMP/SMZ dose, using only 160/800 mg twice daily (332).

Several Gram-negative organisms, which are often ampicillin-resistant, pose problems in therapy that may be resolved, at least in part, by the use of TMP plus SMZ. *S. typhi* strains have been found in Mexico that are resistant not only to chloramphenicol, as previously documented in Southeast Asia (73), but also to ampicillin (407). TMP/SMZ would be expected to be effective against such strains in vivo (if they are susceptible in vitro), as it was against chloramphenicol-resistant, ampicillin-susceptible strains in Southeast Asia and in Mexico (73, 186).

Ampicillin-resistant shigellae were a major problem in Mexico and the southwestern United States. Because patients with ampicillin-resistant organisms continue to have diarrhea and positive stool cultures during ampicillin treatment, and because the resistant shigellae are often susceptible to TMP plus SMZ in vitro (460), several trials were carried out to assess the efficacy of TMP/SMZ in the treatment of shigellosis. The results of these studies suggest that TMP/SMZ is at least as good as ampicillin for the treatment of this disease (92, 387). Unfortunately, TMP-resistant strains of shigella that are also resistant to TMP/SMZ synergism have now been reported from many parts of the world (312).

H. influenzae type b organisms resistant to ampicillin were first noted in the United States in 1974 (264, 534). Since that time, these strains have appeared widely in the United States (487). Because ampicillin-resistant strains are typically susceptible to TMP plus SMZ in vitro, there has been continuing interest in use of TMP/SMZ to treat infections caused by these strains. Although both erythromycin/SMZ (487) and streptomycin/SMZ (350) combinations have also been used, TMP/SMZ has continued to play an important role in the treatment of ampicillin-resistant *H. influenzae* infection.

Other infections in which TMP/SMZ has been efficacious include gonorrhea (22) and infections with multiply resistant *Acinetobacter calcoaceticus*, *Enterobacter*

spp., and *N. asteroides* (332, 399). This combination has also proven useful in the treatment of serious infections due to methicillin-resistant but TMP/SMZ-susceptible strains of *S. aureus* in patients who cannot tolerate vancomycin (444). TMP/SMZ has also been employed successfully in the treatment of infection due to *Listeria monocytogenes* (507).

TMP/SMZ is also useful for the treatment of urinary tract infections due to Gram-negative organisms, especially the Enterobacteriaceae. Its activity is enhanced by increased levels in the urine secondary to renal concentration and, in particular, because levels of TMP (the more active component for most organisms) are increased more than those of SMZ (486).

Additional indications for TMP/SMZ include the prevention of recurrent urinary tract infection (219, 511) and the treatment of chronic prostatitis, because TMP penetrates especially well into prostatic fluid (351). (TMP is concentrated in prostatic secretions to levels three times plasma levels, but SMZ levels in prostatic secretions are only one tenth those found in plasma [585].) TMP/SMZ has also been established as effective for the prophylaxis and treatment of *Pneumocystis carinii* pneumonia (68, 69) and is now widely used for this purpose (99).

Predicting Resistance to Therapy

Problems with TMP/SMZ susceptibility testing are most frequently related to the thymidine content of the testing media, i.e., the TMP MIC has been shown to rise with the thymidine content (279). Similar problems occurred earlier in sulfonamide testing, and in 1945 it was demonstrated that lysed horse blood could improve otherwise unsuitable media (220). Since then it has been shown that lysed horse red cells contain thymidine phosphorylase, which converts thymidine to thymine, thus decreasing its ability to antagonize the TMP/SMZ blockade by approximately 100-fold (68).

For this reason, Bushby (68) has recommended the addition of lysed horse blood to media used for TMP/SMZ testing, unless the thymidine content is known to be less than 0.3 μg/mL. Unsupplemented Mueller-Hinton agar is usually satisfactory (34, 560), but agar lots should be screened for the absence of significant amounts of thymidine or thymine by demonstrating inhibition of control strains of *E. faecalis* by TMP/SMZ disks (34). For susceptibility testing with TMP/SMZ in broth, supplementation of the medium with lysed horse blood or addition of commercially available thymidine phosphorylase usually results in satisfactory performance (386).

Inhibition of Enzymes That Render Antimicrobial Agents Inactive

This section describes antimicrobial combinations in which one drug interferes with either the production or the action of a bacterial enzyme, thus permitting another drug (which would otherwise have been ineffective) to be active.

Mechanism of Action

Bacterial enzymes may exert a protective effect by hydrolyzing a substrate such as penicillin, thus rendering it inactive against the bacterial cell. Two approaches to antimicrobial synergism have attempted to circumvent this mechanism, by (*a*) using another drug to prevent the production of β-lactamase or (*b*) binding the enzyme so tightly that it is not free to act on the susceptible (hydrolyzable) penicillin, thus allowing that penicillin to remain intact and to exert its bactericidal effect on the β-lactamase-producing organism (Fig. 9.15). Inhibitors of an aminoglycoside-modifying enzyme (aminoglycoside-2″-*O*-adenylyltransferase) have also been described and potentiate the activities against enzyme-producing Gram-negative bacilli of aminoglycosides susceptible to modification at the 2″-position (4). A third approach has been the use of a urease inhibitor (acetohydroxamic acid) (382) to prevent the rise in urinary pH associated with urease activity (especially in *Proteus* infections), so that drugs that require acidic urine (methenamine mandelate) (381) might be effective. This urease inhibitor also inhibits growth of *Helicobacter pylori* at high concentrations and appears to augment activities of various antimicrobials against some isolates (434). However, neither the aminoglycoside-modifying enzyme inhibitors nor inhibitors of urease activity have been developed to a level of clinical utility and, therefore, are not discussed further in this chapter.

Laboratory Studies of Synergism by Inhibition of Enzymes That Render Antimicrobial Agents Inactive

Penicillin Combinations Using an Inhibitor β-Lactam to Bind β-Lactamase. A substantial body of literature describes the use of penicillin combinations against β-lactamase-producing strains in vitro. In early studies, these combinations usually involved an inhibitor (less hydrolyzable) penicillin (e.g., cloxacillin) to bind the β-lactamase and a hydrolyzable penicillin (e.g., penicillin G), which would ordinarily have been inactivated by β-lactamase. It has been suggested that four criteria must be met for such combinations to successfully produce synergism (464): (*a*) β-lactamase must be a major factor in the resistance of the organism to penicillin, (*b*) the inhibitor β-lactam must be resistant to the β-lactamase of the organism, (*c*) the inhibitor β-lactam must have a greater affinity for the β-lactamase than does the hydrolyzable penicillin, and (*d*) the inhibitor β-lactam must be relatively ineffective as an antimicrobial against the test strain at the concentration employed (otherwise, it would inhibit and kill the test organism alone).

The failure to demonstrate synergism in early studies with *S. aureus* (467) was attributed to a low affinity of the inhibitor penicillin for staphylococcal penicillinase (464). The affinity of methicillin (the inhibitor penicillin) for staphylococcal β-lactamase is less than 10^{-4} that of benzylpenicillin (403).

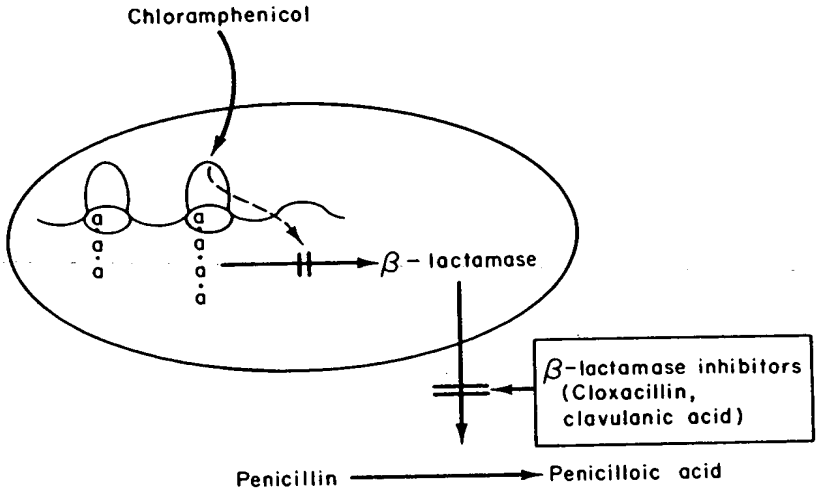


Figure 9.15. Mechanisms of synergism due to inhibition of the production or the action of β-lactamase. Inhibitors of protein synthesis, such as chloramphenicol, act by decreasing the synthesis of β-lactamase. (Clindamycin [not shown] may act by preventing the derepression of inducible β-lactamases.) Inhibitor β-lactams (such as cloxacillin, methicillin, clavulanic acid, tazobactam, and sulbactam) act by binding to bacterial β-lactamases and thus decreasing their ability to inactivate other hydrolyzable β-lactams.

However, study of Gram-negative organisms, particularly *Pseudomonas pyocyanea*, revealed that their β-lactamases have a much greater affinity for methicillin and cloxacillin than for penicillin or ampicillin, thus opening the way for consideration of synergistic penicillin combinations (465). This type of synergism has been reported in vitro against *P. aeruginosa* (466), *E. coli* (525), *Proteus* spp. (404), *Klebsiella* spp. (466), *Enterobacter* spp. (158), and a number of other Gram-negative organisms. Because it requires very high concentrations of methicillin or cloxacillin to produce synergism with penicillins against Gram-negative bacilli, such combinations have been useful only in the treatment of urinary tract infections.

Clavulanic acid, a β-lactam isolated from *Streptomyces clavuligerus*, has also been shown to inhibit the penicillinases produced by a number of bacteria (66, 446). Although it shares a common β-lactam ring with penicillin, it differs structurally in several aspects (Fig. 9.16) (446): (*a*) the sulfur atom of the thiazolidine ring is replaced by an oxygen, producing an oxazolidine ring; (*b*) there is no side chain connected by an amide linkage at position 6 of the β-lactam ring; and (*c*) the two methyl groups at position 2 of the thiazolidine ring are replaced by a β-hydroxylethylidine group. Also shown in Figure 9.16 is the chemical structure of the sulfone sulbactam (150), another β-lactamase inhibitor that has been developed for clinical use. In vitro studies have shown that clavulanic acid and sulbactam inhibit the plasmid-mediated β-lactamases of *S. aureus* and many Enterobacteriaceae but are ineffective against the chromosomal β-lactamases of *P. aeruginosa* and *E. cloacae* and a chromosomally mediated enzyme from *E. coli* (66, 446). The most recently introduced β-lactamase inhibitor, tazobactam, is another penicillinic acid sulfone that is available in fixed combinations with piperacillin (14).

Although none of these inhibitors demonstrates significant antimicrobial activity against most commonly encountered organisms (67, 394, 589), all three drugs considerably extend the spectrum of hydrolyzable penicillins and cephalosporins against a broad range of Gram-negative and Gram-positive organisms possessing (usually plasmid-mediated) β-lactamases susceptible to inhibition by these drugs (14, 150, 288, 300, 431, 446, 497, 591, 592, 594). Synergism between amoxicillin and clavulanate, ticarcillin and clavulanate, ampicillin and sulbactam, or piperacillin and tazobactam, the four fixed-dose β-lactam/β-lactamase inhibitor combinations currently available in the United States, yields useful antimicrobial activity against many strains of *S. aureus*, *B. fragilis*, *H. influenzae*, *K. pneumoniae*, and other Enterobacteriaceae, among others (33, 199, 288, 553). These inhibitors also restore activities of several third-generation cephalosporins against Enterobacteriaceae producing a number of plasmid-mediated, extended-spectrum β-lactamases, most of which are related to TEM or SHV enzymes (245, 411). As stated above, such combinations offer little advantage against most *P. aeruginosa* or *E. cloacae* strains, whose chromosomal β-lactamases are poorly inhibited by these agents. In fact, at high concentrations, clavulanic acid may function as an inducer of these derepressible β-lactamases, hence antagonizing the activities of the intrinsically more active penicillins

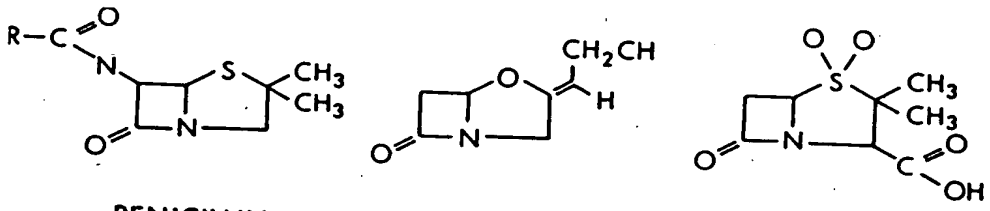


Figure 9.16. Structures of penicillin, clavulanic acid, and sulbactam. Both β-lactamase inhibitors (clavulanic acid and sulbactam) share a double-ring structure and the β-lactam bond in common with penicillin.

against some strains (138). Compounds that inhibit chromosomal β-lactamases of Gram-negative bacteria have been developed, e.g., BRL 42715 (100, 605), but none is available for clinical use at this time.

Inhibition of β-Lactamase Production. Because penicillinases (β-lactamases) are proteins, the use of drugs that inhibit protein synthesis might theoretically inhibit their production enough to render β-lactamase-producing organisms susceptible to penicillin. Therefore, several investigators have examined antimicrobial combinations of inhibitors of protein synthesis (e.g., chloramphenicol) with penicillin in vitro against strains that produce β-lactamase and are thus characteristically penicillin-resistant.

It was demonstrated in 1973 that chloramphenicol could synergize with β-lactam antibiotics against *Klebsiella*, *Enterobacter*, and *Serratia* (322). Because these combinations were more frequently synergistic against strains resistant to β-lactams, it seemed possible that chloramphenicol might exert its effect through inhibition of the protein synthesis required for β-lactamase production. This hypothesis was later supported by a study of Enterobacteriaceae in which the killing-curve technique was used to demonstrate synergism between cephaloridine (12.5 to 500.0 μg/mL) and chloramphenicol (6 μg/mL) against 10 of 10 β-lactamase-producing strains (355). Although chloramphenicol reduced the loss of cephaloridine activity in the medium (measured by bioassay) in that study, β-lactamase was not measured, and chloramphenicol-induced inhibition of β-lactamase production was implied but not proven. The same group of investigators later reported penicillin/chloramphenicol synergism against 17 of 20 β-lactamase-producing strains of *S. aureus* (356). Although high concentrations of chloramphenicol antagonized the inhibitory effect of penicillin, lower concentrations (4 μg/mL) prevented measurable β-lactamase production and produced synergism, as determined by the cellophane-transfer technique (86). More recently, clindamycin was demonstrated to inhibit derepression of the inducible β-lactamases of *E. cloacae* and *P. aeruginosa* (479). Combined with cefamandole (an agent susceptible to hydrolysis by the *E. cloacae* enzyme), clindamycin augmented the in vitro bactericidal activity and in vivo efficacy of the cephalosporin.

Resistance to Synergism by Inhibition of Bacterial Enzymes That Render Antimicrobial Agents Inactive

Combinations Using an Inhibitor β-Lactam to Bind β-Lactamase. As mentioned above, a β-lactam inhibitor that is bound more tightly to β-lactamase than is a hydrolyzable penicillin or cephalosporin may produce synergism against certain organisms when combined with that penicillin or cephalosporin. Inadequate binding of cloxacillin to staphylococcal β-lactamase was a major reason for failure to demonstrate penicillin/cloxacillin synergism against *S. aureus* (467). Such combinations (ampicillin/cloxacillin) also synergistically inhibit Gram-negative organisms, but poor penetration of the latter drug into these organisms severely limits the usefulness of such combinations (52). Obviously, addition of clavulanic acid, sulbactam, or tazobactam to penicillins or cephalosporins offers no advantage against strains resistant to the latter based on production of enzymes (primarily chromosomal) against which the inhibitors are inactive (66). Several novel, plasmid-mediated β-lactamases that are resistant to inhibition by the currently available inhibitors have been reported (245, 412). One of these, designated MIR-1, is mediated by a gene with DNA sequence similarity to that mediating production of chromosomal cephalosporinase of *E. cloacae*, which appears to explain both the origin of this enzyme and its resistance to inhibition (412). Resistance to β-lactam/β-lactamase combinations (e.g., amoxicillin/clavulanate) among some strains of *E. coli* has been attributed to hyperproduction of TEM-type β-lactamases (against which clavulanate is usually active) encoded by small multicopy plasmids (343).

Inhibition of β-Lactamase Production. The use of inhibitors of protein synthesis to inhibit β-lactamase synthesis and thereby potentiate activity of an otherwise hydrolyzable β-lactam has not proven feasible for general use. Therefore, resistance mechanisms are largely theoretical and would include: (*a*) emergence of resistance to the inhibitor of protein synthesis, rendering it ineffective in inhibiting β-lactamase synthesis; and (*b*) manifestation of antagonistic interactions arising from interference with β-lactam-induced lysis, either by direct inhibition (by chloramphenicol) of cellular autolysins or by gen-

eration of a population of nongrowing cells that are less susceptible to penicillin-induced lysis (533).

Clinical Studies of Synergism by Inhibition of Bacterial Enzymes That Render Antimicrobial Agents Inactive

Penicillin Combinations Using an Inhibitor β-Lactam to Bind β-Lactamase. The use of older β-lactamase-resistant penicillins (e.g., cloxacillin) to synergistically enhance the activities of hydrolyzable penicillins (e.g., ampicillin) against Gram-negative bacilli was severely constrained by the fact that effective concentrations of the former often exceeded levels readily achievable in serum (158). Therefore, use of such combinations was limited to treatment of urinary tract infections (464, 466). In contrast, combinations of clavulanic acid with amoxicillin or ticarcillin, of sulbactam with ampicillin, and of tazobactam with piperacillin have proven efficacious against infections caused by a wide variety of susceptible pathogens and are currently approved for clinical use in the United States (14, 199, 589). Such combinations not only provide greater potency than the penicillin alone against some organisms (e.g., *B. fragilis*) but also extend the antibacterial spectrum to bacteria usually resistant to clinically achievable concentrations of these penicillins alone (e.g., *K. pneumoniae* and *S. aureus*). One novel approach was to combine ampicillin with sulbactam through an ester linkage to create a new compound (sultamicillin) that, after in vivo hydrolysis to the parent drugs, produced higher serum levels after oral administration than observed with either of the single agents alone (142).

Inhibition of β-Lactamase Production. Clinical trials have not been carried out to test the use of inhibitors of protein synthesis to block penicillinase production and thus render β-lactamase-producing organisms penicillin-sensitive. The major theoretical reason for the lack of such trials is probably the narrow range of chloramphenicol concentrations over which synergism has been observed in vitro, with documented antagonism at slightly higher levels (356). It seems unlikely that one could approach such precise regulation of antibiotic levels clinically, even with continuous intravenous infusion, which would be prohibitively expensive and complex. The major practical reason that such studies have not been forthcoming is probably the availability of several newer drugs resistant to the β-lactamases of concern.

Combinations of Agents That Act on the Bacterial Cell Wall

Although the penicillin combinations described earlier are combinations of agents that, in principle, have activity against bacterial cell wall synthesis, the inhibitor penicillin in those combinations is primarily functioning to bind β-lactamase and probably does not affect cell wall synthesis of most organisms at clinically relevant concentrations. In this section, we consider combinations in which both antimicrobials appear to act on the bacterial cell wall.

It has been known for many years that penicillin inhibits bacterial cell wall synthesis, particularly at the cross-linking step (46). There has been interest in combinations of penicillin with drugs that act earlier than the cross-linking step of cell wall synthesis (e.g., β-chloro-D-alanine, phosphonic acid, or vancomycin) or with β-lactams that have morphologic effects on bacteria different from those seen with penicillin (e.g., amdinocillin, formerly known as mecillinam), resulting from inhibition of different penicillin-binding proteins (PBPs) (413) than those that are the primary targets of penicillin. Examples of such combinations, few of which are clinically important, are given in this section.

Mechanism of Action

β-Chloro-D-alanine plus Penicillin. β-Chloro-D-alanine (an analog of D-alanine) probably acts by competitively inhibiting the synthesis and/or attachment of the terminal D-alanine dipeptide to the pentapeptide necessary for production of the bacterial cell wall (337). The enzymes that have been implicated as the sites of this inhibition are alanine racemase (which converts L-alanine to D-alanine) and D-glutamate-D-alanine transaminase (which transfers the D-alanine-D-alanine dipeptide to the *N*-acetyl-uridine diphosphate peptide) (504). Thus, the synergism observed with β-chloro-D-alanine plus penicillin is presumably the result of activity exerted at two points in the sequence of cell wall synthesis: (*a*) decreased production and attachment of the D-alanine dipeptide due to β-chloro-D-alanine and (*b*) inhibition of the later cross-linking step due to penicillin.

Phosphonic Acid Derivatives plus β-Lactams. Phosphonic acid derivatives, such as fosmidomycin, alafosfalin, and fosfomycin, also inhibit the early steps of cell wall synthesis. Thus, mechanisms of synergy with β-lactams are presumably analogous to those of β-chloro-D-alanine (discussed in "β-Chloro-D-alanine plus Penicillin"). These drugs have been shown to inhibit alanine racemase and uridine 5′-diphosphate *N*-acetylmuramyl-L-alanine synthetase (alafosfalin) (19) and phosphoenolpyruvate synthetase (fosmidomycin and fosfomycin) (254, 397). Fosfomycin may also affect the synthesis of PBPs (207, 548), providing yet another possible mechanism of interaction with β-lactams.

Amdinocillin plus Other β-Lactams. Amdinocillin (formerly known as mecillinam) is a β-lactam antibiotic that differs in structure from penicillin in one major respect: it has an amidino side chain at the 6-position, in contrast to the acylamino side chain at the 6-position of the more traditional penicillins (Fig. 9.17) (323). Exposure of bacteria to amdinocillin results in the formation of large spherical cells that lyse without formation of spheroplasts (205, 323, 413). These observa-

tions suggest a different mode of action for amdinocillin than for penicillin, which typically produces filamentous forms of *E. coli* at low concentrations, bulges in bacilli at intermediate concentrations, and finally spheroplasts (which lyse unless maintained in a hypertonic medium) at high concentrations (Fig. 9.18) (509). Additional evidence for a different mechanism of action for amdinocillin was provided by the observation that it does not inhibit the murein transpeptidase (cross-linking enzyme), D-alanine carboxypeptidase I, or murein endopeptidase activities of *E. coli*, all of which are inhibited by ampicillin (413). With the development of techniques to electrophoretically separate PBPs of the bacterial cytoplasmic membrane, it became clear that binding of amdinocillin to its primary target—PBP-2 in *E. coli*—accounts for the morphologic changes mentioned above (508, 510, 532). Because most of the other penicillins and cephalosporins used clinically bind preferentially to other PBPs (532), combination of amdinocillin with β-lactams acting at complementary target sites might result in synergistic antibacterial interactions.

Vancomycin Combined with Penicillins and Cephalosporins. Although amdinocillin/penicillin combinations are probably the best example of synergism due to combinations of agents that act on the bacterial cell wall, combinations of vancomycin with penicillins and cephalosporins could act by a similar mechanism (127). Because vancomycin acts earlier than the cross-linking step of cell wall synthesis (it inhibits the synthesis of peptidoglycan) (347), its interaction with penicillins or cephalosporins could be classified either as sequential inhibition of a common biochemical pathway or (as we have done) as synergism due to a combination of agents that act on the bacterial cell wall.

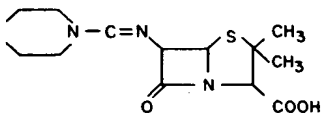
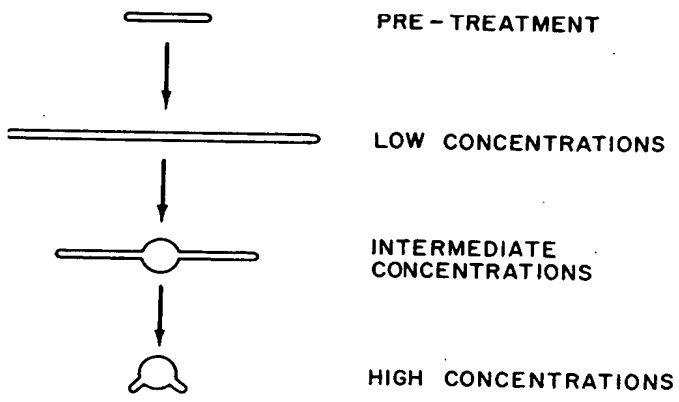


Figure 9.17. Structure of amdinocillin.

Figure 9.18. Morphologic effects of increasing concentrations of penicillin on *E. coli*. (Reproduced with permission from Spratt [509].)



Laboratory Studies Using Combinations of Agents That Act on the Bacterial Cell Wall

β-Chloro-D-alanine plus Penicillin. Using a *Salmonella typhimurium* strain resistant to penicillin, synergism has been demonstrated with penicillin and concentrations of β-chloro-D-alanine that are ineffective when used alone (4 to 8 μg/mL) (504).

Phosphonic Acid Derivatives plus β-Lactams. Alafosfalin plus amdinocillin or ampicillin produce synergism against a variety of Enterobacteriaceae (19). Similarly, fosmidomycin plus penicillin or cephalosporin combinations are synergistic against many *E. coli*, *K. pneumoniae*, and *S. marcescens* (397). Combinations of fosfomycin with a variety of β-lactams have yielded synergistic inhibition of a wide range of organisms, including Enterobacteriaceae (426), *Pseudomonas* (526), and staphylococci (6). However, because bacterial strains resistant to fosfomycin can be selected easily, both in vitro and in vivo (529), it is not clear in most cases whether a beneficial interaction of two drugs results primarily from true synergism or from the suppression of resistant clones. Combination of fosfomycin with daptomycin, a cyclic lipopeptide antibiotic (included here for convenience, although it is not a β-lactam) active at the level of cell wall synthesis, has resulted in synergism against *E. faecalis*, including strains demonstrating high-level resistance to gentamicin (453). Fosfomycin induces alterations in PBP patterns in *S. aureus*, resulting in variable, concentration-dependent interactions with oxacillin (384). Alterations in PBP patterns upon incubation in fosfomycin have also been demonstrated with enterococci, which has been suggested as a mechanism of enhanced activity when this drug is combined with penicillin (207).

Amdinocillin plus Other β-lactams. A number of reports have described the action of amdinocillin on bacterial cells. Susceptibility studies suggest that amdinocillin alone is active in vitro (MIC of ≤6.3 μg/mL) against many Enterobacteriaceae (including *E. coli*, *Klebsiella*, *Enterobacter*, *Salmonella*, and *Shigella*). Resistant organisms include *P. aeruginosa*, *Acinetobacter*,

Serratia, *Proteus*, *Providencia*, *H. influenzae*, most Gram-positive cocci and bacilli, and the Gram-negative anaerobes (388). The morphologic effects of amdinocillin and bacterial structure have been reexamined by several investigators. Most reports describe an initial change from typical Gram-negative rods to ovoids or ellipses, followed by the appearance of large spherical forms (diameter of 5 μm) (544) and then by lysis in 2 to 3 hours (354) unless the cells are protected by a hypertonic medium (203). In the initial in vitro studies, it was not clear why amdinocillin could inhibit the growth of β-lactamase-producing Enterobacteriaceae, because it was hydrolyzed by several of these enzymes. It now appears that the explanation may be a low affinity of those β-lactamases for amdinocillin (454).

In these and other studies with amdinocillin, different laboratory methods may produce apparently conflicting results. Because amdinocillin tends to cause the production of large spherical forms, the optical density (turbidity) of the culture may remain the same or increase while the number of CFU per milliliter decreases (Fig. 9.19) (545). Therefore, although a decrease in optical density with amdinocillin is probably meaningful, the significance of a stable or rising value would need to be corroborated by determining the number of CFU per milliliter.

In vitro combinations of amdinocillin with other β-lactams have demonstrated synergism against *E. coli* (389, 545), *Klebsiella* (25, 389), *Enterobacter* (389), *Citrobacter* (390), indole-negative *Proteus* (25, 389), *Shigella* (390), *Salmonella* (390), and other Gram-negative aerobes, especially Enterobacteriaceae, which are typically susceptible to amdinocillin. Synergism is usually not seen against Gram-positive cocci (streptococci or staphylococci), Gram-positive bacilli (*Clostridia* or *Listeria*), indole-positive *Proteus* (389), or Gram-negative anaerobes (389), which are generally resistant to amdinocillin alone (see above). Although studies of amdinocillin/β-lactam synergism have usually employed amdinocillin/ampicillin combinations, the use of cloxacillin (42), amoxicillin (389), carbenicillin (25), cephalothin (390), cephradine (263), and other β-lactams with amdinocillin has yielded similar results.

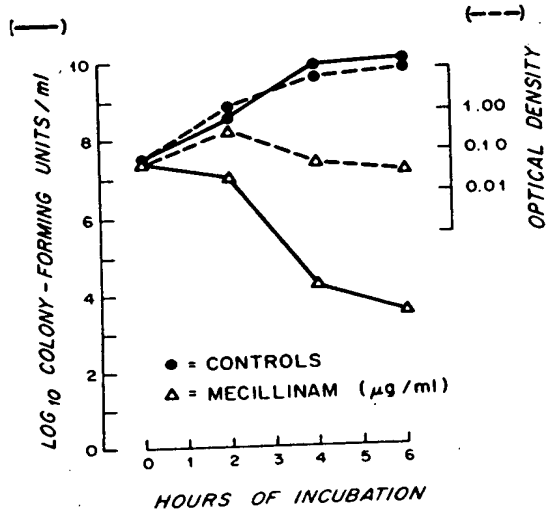


Figure 9.19. Effect of amdinocillin on the growth of *E. coli*, as measured by colony counts (——) and by optical density (– – – –). (Adapted from Tybring and Melchior [545], with permission.)

More recent studies have provided support for the cooperative interaction between β-lactams at the level of specific high-affinity targets (PBPs) in augmenting bacterial death and lysis. Gutmann et al. (211) demonstrated rapid bactericidal effects against *E. coli* with combinations of amdinocillin (PBP-2-specific) plus aztreonam (PBP-3-specific) at drug concentrations that were only bacteriostatic individually. The relevance of PBP binding (and, hence, inactivation of these enzymes) in this phenomenon was further supported by studies with temperature-sensitive PBP-2 or PBP-3 mutants. Under conditions in which one or the other of these was not expressed, addition of the drug specifically binding the complementary PBP resulted in cell lysis comparable to that seen when the two antibiotics were combined. Another example of bactericidal synergism based on cooperative effects of β-lactams binding preferentially to different PBP targets was provided by the work of Tuomanen et al. (542). In that study, fine balance between inhibition of PBP-3 (by aztreonam) and of PBP-1a (by the cephem cefsulodin) produced a bactericidal effect against *E. coli* without cell lysis, whereas the individual agents caused filamentation without cell death or death with cell lysis, respectively (542).

Studies by Sanders et al. (480) have revealed another possible mechanism by which amdinocillin may potentiate the activities of other β-lactams. Exposure of Gram-negative bacilli to amdinocillin caused leakage of β-lactamase into the culture medium. It can be postulated that defects in the cell envelope leading to leakage may also facilitate penetration of other β-lactams or may render the cell more vulnerable to β-lactams by virtue of diminished inactivating enzyme in the periplasmic space.

Study of synergy with amdinocillin plus other β-lactams has suggested the potential importance of the minimal concentration of antimicrobial necessary to produce either a change in bacterial morphology detectable by light or electron microscopy or a 10-fold reduction in growth (319). This concentration, which is less than the MIC, has been termed the minimal antibacterial or minimal active concentration (319), although it was initially described as the lowest concentration to produce a change in morphology (315). A synergistic effect against *P. mirabilis* and *E. coli* has been demonstrated by the killing-curve technique with combinations of amdinocillin and ampicillin at concentrations 10^{-3} and 10^{-2} of their respective MICs (315). The minimal concentrations

necessary to produce this synergistic effect corresponded to the minimal antibacterial concentrations of each drug alone (Fig. 9.20).

Other β-Lactam Combinations. Several authors have reported synergism between different β-lactams against various organisms. These include imipenem or meropenem with other β-lactams against methicillin-resistant *S. aureus* (346, 406, 524) and imipenem plus ampicillin (55) or amoxicillin plus cefotaxime (333) against enterococci. A special case of double β-lactam interaction arises with the use of cefotaxime. The desacetyl metabolite of this drug is biologically active and its combination with the parent compound sometimes results in apparently synergistic interactions against staphylococci (77), anaerobes (77, 341, 370), and Gram-negative bacilli (341, 370).

Vancomycin in Combination with Penicillins or Cephalosporins. Synergistic results have been observed with high concentrations of vancomycin plus β-lactams (by the checkerboard technique) against three Gram-negative organisms. Vancomycin/carbenicillin synergism was observed with 19 of 25 *P. aeruginosa* strains, 12 of 25 *K. pneumoniae* strains, and 19 of 25 *S. marcescens* strains; vancomycin/cephalothin synergism was noted with 12 of 25 *P. aeruginosa* strains, 19 of 25 *K. pneumoniae* strains, and 15 of 25 *S. marcescens* strains (127). Against *S. aureus* synergistic interactions have been recorded for combinations of vancomycin with cefpirome (491) and for vancomycin or teicoplanin combined with imipenem (32).

Combinations of glycopeptides with β-lactams can result in bacteriostatic synergism against vancomycin-re-

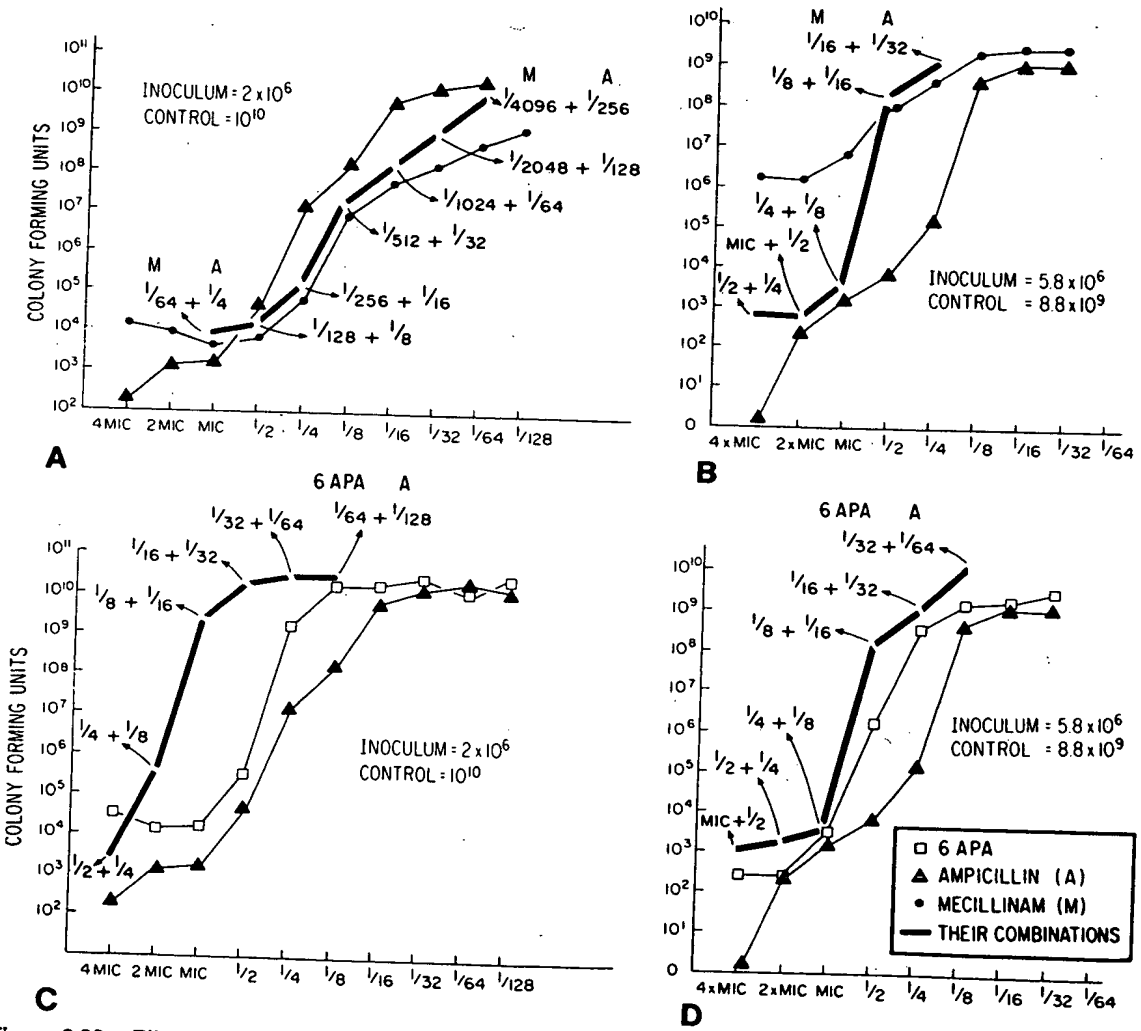


Figure 9.20. Effect of ampicillin (*A*), amdinocillin (mecillinam [*M*]), 6-aminopenicillanic acid (*6 APA*), and their combinations on the viability of *P. mirabilis* (**A** and **D**) and *E. coli* (**B** and **C**).

sistant enterococci (45, 296). Such interactions do not occur predictably nor are they necessarily seen at clinically achievable drug concentrations (84). It has been hypothesized that the novel peptidoglycan synthesized following induction of the vancomycin resistance system cannot be adequately processed by enterococcal PBPs with low affinity for penicillin, rendering the cells hypersusceptible to the β-lactam (5). In animal models of infection with vancomycin- and penicillin-resistant *Enterococcus faecium*, combinations of vancomycin or teicoplanin with penicillin or ceftriaxone have demonstrated synergistic inhibitory activity, which in turn results in synergistic bactericidal activity when gentamicin is added (79–81).

Resistance to Synergism by Combinations of Agents That Act on the Bacterial Cell Wall

Resistance to Synergism by β-Chloro-D-alanine plus Penicillin. There has been only limited in vitro study of penicillin plus β-chloro-D-alanine synergism, and resistant strains have not been described (504).

Resistance to Phosphonic Acid Derivative plus β-Lactam Synergism. The presently available studies suggest that Gram-negative bacilli susceptible to phosphonic acid compounds alone are less susceptible to phosphonic acid derivative/β-lactam synergism (397). Our studies with the fosfomycin/daptomycin combination against enterococci revealed bactericidal synergism only when the daptomycin concentration employed yielded a relatively weak bactericidal effect as a single agent (453).

Resistance to Amdinocillin plus β-Lactam Synergism. Data from a number of laboratories suggest that strains that are resistant to amdinocillin in vitro are usually also resistant to amdinocillin/β-lactam synergism. Mechanisms of such resistance may include alterations in PBP-2 targets (e.g., enzymes with reduced affinity for the drug), alterations at the level of the outer membrane of Gram-negative bacilli resulting in diminished penetration of drug, or elaboration of β-lactamases capable of hydrolyzing the drug. In the laboratory, exposure of bacteria to amdinocillin in culture medium or urine can also permit the selection of phenotypically resistant variants that revert to normal morphology, antibiotic susceptibility, and generation time when returned to amdinocillin-free medium (9). Three morphologic types of phenotypically resistant variants have been described (348).

Resistance to Synergism by Vancomycin in Combination with Penicillins or Cephalosporins. The mechanism by which some strains of *Pseudomonas*, *Klebsiella*, and *Serratia* are resistant to vancomycin/β-lactam synergism in vitro is not known. However, isolates that satisfy the criteria for synergism by the checkerboard technique (reduction of the MIC to less than one fourth of its original value) with the addition of a second drug tend to have higher vancomycin MICs (≥5000 μg/mL) than do isolates that are resistant to synergism (MICs of 1250 to 2500 μg/mL) (127). These data are compatible with an absolute requirement for high levels of vancomycin (perhaps to overcome a permeability barrier) in order to produce vancomycin/β-lactam synergism. It is thus possible that organisms with lower vancomycin MICs fail to fulfill the criteria for synergism because one fourth their vancomycin MIC is a concentration at which only small amounts of vancomycin penetrate the cell wall.

In animal experiments with vancomycin-resistant enterococci that demonstrate susceptibility to glycopeptide/β-lactam inhibitory synergism, colonies resistant to the synergistic interaction can be recovered among surviving bacteria at the completion of treatment (81). Studies of mutant colonies resistant to synergism sometimes demonstrate alterations in PBPs, but for other strains mechanisms of resistance remain obscure (210).

Clinical Studies of Synergism by Combinations of Agents That Act on the Bacterial Cell Wall

β-Chloro-D-alanine plus Penicillin. This approach to antimicrobial synergism has not achieved clinical application. Another analog of alanine (cycloserine) has been used in the treatment of tuberculosis, but its use has been limited due to toxicity. Confusion, seizures, psychosis, peripheral neuropathy, and hepatotoxicity have all followed the use of this agent (518). It has not been used clinically in combination with penicillin.

Amdinocillin plus β-Lactam Synergism. Several controlled trials of amdinocillin/β-lactam combined therapy have been reported. An early study suggested that a combination of pivmecillinam (the pivaloyloxymethyl ester of amdinocillin) plus amoxicillin was superior to amoxicillin alone when given three times daily for 10 days to patients with purulent exacerbations of chronic bronchitis (436). The authors suggested that the mechanism of the more rapid general improvement and more rapid conversion of purulent mucoid sputum might be antimicrobial synergism against *H. influenzae* (208, 588), which is normally resistant to amdinocillin alone. File and Tan (167) studied amdinocillin plus cefoxitin versus cefoxitin alone in the treatment of mixed soft-tissue infections, including diabetic foot infections. Although a higher percentage of patients responded satisfactorily to combined therapy (90 versus 71%), the number of patients studied was too small for the differences to reach statistical significance (167). Sattler et al. (482) compared amdinocillin plus β-lactam therapy with amdinocillin plus aminoglycoside treatment for serious Gram-negative infections. The therapeutic responses were similar in the two groups (482). Amdinocillin plus cefoxitin combinations have also been used successfully in the treatment of urinary tract infections due to multiply resistant *S. marcescens* (559).

Although amdinocillin was approved for clinical use in the United States, its application was limited by the relatively narrow spectrum of the drug itself, by the in-

ability to predict definitively, without actual testing in vitro, whether synergism would occur against a given isolate, and by the availability of highly effective alternative agents (139).

Vancomycin Combined with Penicillins or Cephalosporins. The levels of vancomycin required to produce synergism with β-lactams against Gram-negative bacilli in vitro (78 to 2500 μg/mL) (127) are equal to or greater than those that have been related to serious ototoxicity (80 to 100 μg/mL) (180). Therefore, clinical trials of this combination against Gram-negative organisms will undoubtedly not take place. Synergism between vancomycin and β-lactams against vancomycin-resistant enterococci can occur at clinically attainable concentrations of both drugs. However, the survival of mutant clones resistant to synergism after treatment in animal models (81) sheds doubt on the applicability of such regimens. Human studies with such regimens have not been reported.

Predicting Resistance to Therapy

Predicting resistance to synergism in vitro with combinations of cell wall-active agents is not an issue for β-chloro-D-alanine plus penicillin or for vancomycin plus β-lactam combinations against Gram-negative organisms, because neither of these is likely to be useful clinically, for the reasons outlined above. For the amdinocillin plus β-lactam combination, it should be possible to predict which organisms will likely fail to respond synergistically by their MICs for amdinocillin alone, because MICs of greater than 6.3 μg/mL have been associated with resistance to amdinocillin plus β-lactam synergism in vitro (see above). Among isolates fully susceptible to the drug, the added value of synergism attained by combination with another β-lactam is questionable. Synergism between glycopeptides and β-lactams against vancomycin-resistant enterococci is not predictable from susceptibility levels for the individual agents. Some methods of detecting synergistic interactions are quite simple (296), but these have not been standardized for clinical use.

Use of Agents Active on the Cell Wall to Enhance the Uptake of Aminoglycosides

Although resistance to antimicrobials may be the result of drug-inactivating enzymes (41, 115) or an insensitive target site (e.g., a ribosome resistant to aminoglycosides) (608, 609), it may also be due to a permeability barrier. In such situations, a given drug could be active if another agent altered the permeability of the bacterial cell in order to permit its entry. It has been postulated that agents that act on the cell wall may enhance the entry of aminoglycosides in this manner in a number of bacterial species. Amphotericin B similarly facilitates the entry of 5-fluorocytosine and other agents into fungi (353). In this section, we consider combinations of agents active on the cell wall with aminoglycosides.

Mechanism

Work on enterococci (*E. faecalis*) has shown that the uptake of ^{14}C-labeled streptomycin is significantly increased in the presence of penicillin (Fig. 9.21) (365).

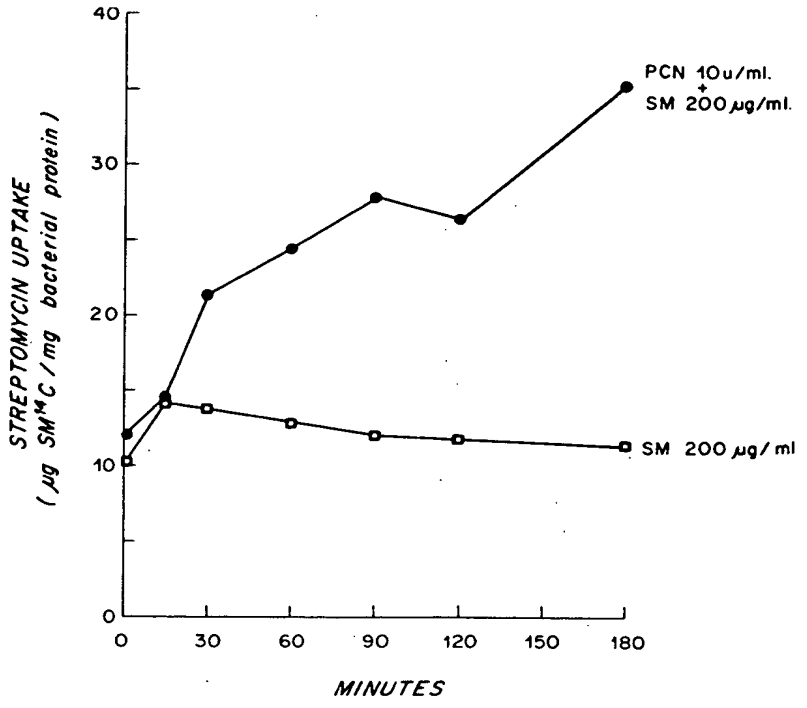


Figure 9.21. Effect of penicillin (*PCN*) on the uptake of [^{14}C]streptomycin (*SM*) by *Enterococcus faecalis*. (Reproduced with permission from Reference 365.)

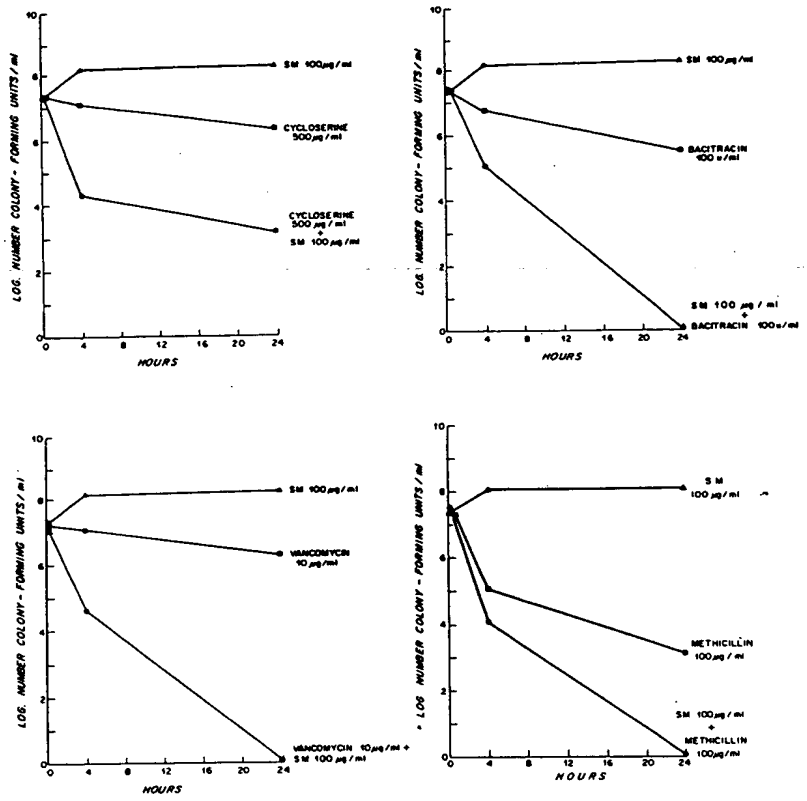


Figure 9.22. Effect of cycloserine, bacitracin, vancomycin, or methicillin plus streptomycin (*SM*) against *E. faecalis*. (RC Moellering Jr, unpublished observations.)

Furthermore, this effect is not specific for penicillin and is also seen with other agents acting on the cell wall (cycloserine, bacitracin, and vancomycin), all of which presumably act similarly to permit increased entry of aminoglycosides (365). To the extent that the aminoglycoside is active intracellularly—that is, unless the drug is rendered ineffective by aminoglycoside-modifying enzymes or unless the organism is resistant at the ribosomal level (139)—such combinations usually exert a synergistic effect. Enhancement of intracellular uptake of aminoglycosides in the presence of cell wall-active agents, with resulting bactericidal synergism, has also been documented in viridans streptococci (596) and *S. aureus* (604). As early as 1962, Plotz and Davis (438) demonstrated enhanced bactericidal activity against Gram-negative bacilli, with augmented uptake of [^{14}C]streptomycin, in the presence of penicillin. Similar mechanisms have more recently been demonstrated to be operative in *P. aeruginosa* (357). Mechanisms of interaction are potentially more complicated in Gram-negative organisms, however. For example, in Gram-negative bacilli, exposure to aminoglycosides may produce relative permeabilization of the outer cell envelope to some β-lactam antibiotics (217, 218).

In both *E. faecalis* and *E. coli*, sequential exposure to ampicillin followed by an aminoglycoside (amikacin) results in a greater bactericidal effect than when the drugs are applied in the reverse order (317), providing support for the primary effect of the cell wall-active agent in the initiation of bactericidal synergism when the agents are administered simultaneously.

Laboratory Studies Using Agents Active on the Cell Wall to Enhance the Uptake of Aminoglycosides

Because many different inhibitors of cell wall synthesis have been combined with aminoglycosides against a variety of bacteria, the discussion of laboratory studies has been organized by the bacteria that were being tested.

Enterococci. The original observation of penicillin/aminoglycoside synergism was probably made in 1947 (239). Subsequently, many other investigators have demonstrated a synergistic effect of penicillin plus various aminoglycosides against enterococci (75, 242, 246, 284, 366, 368, 369).

The cell wall-active agents that enhance the uptake of ^{14}C-labeled streptomycin by enterococci (e.g., penicillin, cycloserine, bacitracin, vancomycin, and ethylenediaminetetraacetic acid) (365) also produce synergism against enterococci when combined with streptomycin (Fig. 9.22) (367). Thus, the present model of penicillin/aminoglycoside synergism in enterococci assumes that the role of agents active on the cell wall is to facilitate the entry of the aminoglycoside (Fig. 9.23). A number of

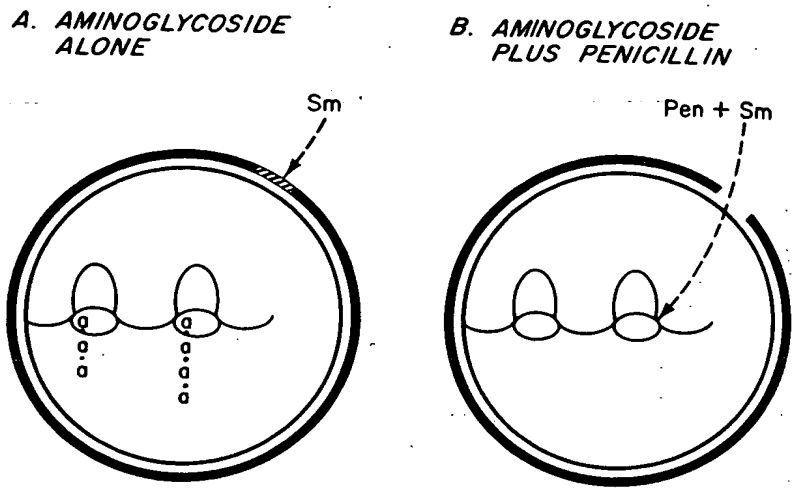


Figure 9.23. Mechanisms of penicillin plus aminoglycoside synergism against enterococci. Based on studies of the uptake of [^{14}C]streptomycin (*Sm*) (see Fig. 9.21), aminoglycosides appear to be unable to enter the enterococcal cell or to reach its ribosomes in the absence of penicillin (*Pen*) (or other agents that act on the bacterial cell wall).

penicillins, including ampicillin, carbenicillin, and penicillin G, have been shown to produce synergism with aminoglycosides against enterococci (242, 310). Among the semisynthetic β-lactamase-resistant penicillins, the available data suggest that the ability to produce synergy in vitro (in broth) with aminoglycosides correlates with the effectiveness (as judged by the MIC) of the semisynthetic penicillin alone against enterococci. Thus, nafcillin is most effective, and oxacillin is more effective than methicillin (190, 563). In addition to these killing-curve studies, oxacillin/gentamicin synergism against enterococci has been shown by the checkerboard technique (338). (The latter technique, however, is not currently recommended for documentation of bactericidal synergism against enterococci [462].)

A number of aminoglycosides have been demonstrated to produce synergism in combination with penicillins against enterococci. These include streptomycin, kanamycin, gentamicin, tobramycin, netilmicin, sisomicin, and amikacin (75, 242, 284, 366, 368, 369).

Other Streptococci. Combinations of penicillin and streptomycin have been shown to be synergistic against most strains of viridans streptococci in vitro by the killing-curve technique (240, 590) and by the checkerboard method (132). Group B streptococci are likewise killed more rapidly in vitro by penicillin (or ampicillin) plus gentamicin than by either drug alone (23, 130, 483).

Staphylococci. Enhanced activity was demonstrated by the time-kill method when nafcillin or oxacillin was combined with gentamicin, tobramycin, netilmicin, or sisomicin against clinical isolates of methicillin-susceptible *S. aureus* (469, 562, 564). In one study, the enhanced activity against *S. aureus* observed with the addition of a β-lactam to an aminoglycoside fulfilled the criteria for synergism (by the killing-curve technique) most frequently for nafcillin/tobramycin (29%) and slightly less frequently for other combinations, including oxacillin/sisomicin (23%), nafcillin/gentamicin (20%), nafcillin/netilmicin (20%), oxacillin/netilmicin (20%), and nafcillin/sisomicin (11%) (562, 564). A synergistic effect by the killing-curve method has been reported for eight of eight penicillinase-producing *S. aureus* strains with nafcillin (5 μg/mL) plus gentamicin (2 or 0.5 μg/mL) (469). Using the checkerboard technique and a reduction of the FIC index to ≤0.5, synergism has likewise been demonstrated with penicillin plus gentamicin against five of six penicillin-susceptible *S. aureus* strains (515). A synergistic effect against methicillin-resistant *S. aureus* (determined by the killing-curve technique) has been demonstrated for combinations of cephalothin (10 μg/mL) plus kanamycin (20 μg/mL) (62) and for high concentrations (50 μg/mL) of either oxacillin or cephalothin plus gentamicin (5 μg/mL) (268).

It is worth noting, however, that beneficial interactions between β-lactams and aminoglycosides against *S. aureus* may also be attributable to other mechanisms. Specifically, such combinations may suppress the late regrowth of colonies seen by time-kill studies when aminoglycosides are tested as single agents (139), which has been associated with decreased susceptibility to bactericidal effects of the aminoglycoside among surviving colonies (306).

Other Gram-Positive Organisms. Bactericidal synergism between β-lactam antibiotics and aminoglycosides against *L. monocytogenes* has been recognized for years (363). Such interactions can be demonstrated by time-kill methods using subinhibitory concentrations of aminoglycosides and inhibitory concentrations of the β-lactams, which are typically bacteriostatic against *Listeria* (144, 326).

Combinations of β-lactams with aminoglycosides have also produced bactericidal synergism against some strains of pathogenic *Corynebacteria* spp. (363). Synergism generally cannot be demonstrated against strains of JK corynebacteria that are resistant to aminoglycosides (MIC of more than 128 μg/mL), but resistance to penicillin only may not always preclude synergism (363). Against this group of organisms, bactericidal synergism

can also be shown between aminoglycosides and the cell wall-active drugs vancomycin and daptomycin (505).

Pseudomonas. Pseudomonads can pose a major clinical problem because of their ability to cause life-threatening infection in immunocompromised hosts and other seriously ill patients (48), and because they are frequently resistant to multiple drugs (3). Thus, when gentamicin and carbenicillin became available, a number of investigators examined the activity of this combination against clinical isolates of *Pseudomonas* in vitro. Following the initial description of gentamicin/carbenicillin synergism in 1967 (59), several laboratories reported a synergistic effect of this combination against *P. aeruginosa* by checkerboard or agar diffusion techniques (135, 276, 374, 500, 503, 512, 599). Killing-curve studies have also shown synergism of this combination against a number of *P. aeruginosa* strains (503, 512). This combination is also synergistic against many clinical isolates of *A. calcoaceticus* var. *anitratus* (188, 376, 377).

Subsequently, ticarcillin, piperacillin, azlocillin, aztreonam, and mezlocillin have been found to enhance the activity of gentamicin and other aminoglycosides against *P. aeruginosa* (10, 101, 139, 557). As judged by their MICs, ticarcillin is approximately twice as active as carbenicillin against *P. aeruginosa*, mezlocillin is approximately as active as ticarcillin (136, 414, 557), and piperacillin and azlocillin are two to four times as active as mezlocillin (145, 527). Similarly, aminoglycosides other than gentamicin, including tobramycin, amikacin, and sisomicin, produce synergism with these β-lactams by the checkerboard technique (101, 272, 275, 278, 340). Rand et al. (445) have called attention to the lack of reproducibility of the microdilution checkerboard method for determining β-lactam/aminoglycoside interactions against *P. aeruginosa* and recommend establishing consistency of test results in replicate determinations if that method is used. Furthermore, results derived from checkerboard methods of determining synergism between antipseudomonal β-lactams and aminoglycosides against *Pseudomonas aeruginosa* may not correlate with those based on time-kill curve techniques (77a).

Klebsiella. *Klebsiella* strains are usually susceptible to the cephalosporins but resistant to ampicillin and carbenicillin (501). Utilizing the checkerboard technique, one study found that synergism was produced by cephalothin plus gentamicin against 73% of 61 strains of *Klebsiella* but by ampicillin and gentamicin against only 21% of 61 strains (270). The combination of cefazolin plus amikacin was found to be synergistic, by both killing-curve and checkerboard techniques, against 11 of 20 *Klebsiella* strains studied (274). Another study utilizing the checkerboard technique demonstrated synergism with cephalothin plus gentamicin, kanamycin, or amikacin against most (69 to 95%) of the 38 *Klebsiella* isolates tested (112). Among studies with newer agents active against *Klebsiella* spp., amikacin plus imipenem (196), ceftazidime (196), or cefotaxime (191) resulted in synergism against 5 to 83% of strains tested. This wide range in outcome undoubtedly reflects differences in strain collections and/or methods used (checkerboard versus time-kill).

Other Enterobacteriaceae. Numerous studies have documented the synergistic potential of older β-lactams (e.g., ampicillin, carbenicillin, cephalothin, etc.) plus aminoglycosides against many strains of *E. coli* (169, 270), *Proteus* spp. (136, 169, 270, 272, 501), and *Enterobacter* spp. (168, 270). For example, by time-kill methods, 46 to 48% of *Enterobacter* were susceptible to ampicillin/gentamicin or cephalothin/gentamicin synergism (270). Several more-recent studies utilizing similar techniques have noted synergism between several extended-spectrum acylaminopenicillins or third-generation cephalosporins and aminoglycosides (often amikacin or gentamicin) against 52 to 90% of Enterobacteriaceae tested (139). A slightly lower frequency of synergistic interactions (16 to 70%) was reported by studies employing checkerboard (MIC) techniques.

Mechanism of Resistance

Enterococci. Following the original descriptions of penicillin/streptomycin synergism, it gradually became apparent that not all enterococci were synergistically killed by this combination. The explanation for this discrepant behavior among clinical isolates of enterococci (536) was not clear until 1970, when two groups of investigators pointed out that isolates with high-level streptomycin resistance (MIC of more than 2000 μg/mL) were resistant to penicillin/streptomycin synergism and that strains without high-level streptomycin resistance (MIC of ≤2000 μg/mL) were susceptible to penicillin/streptomycin synergism (366, 512). Although the correlation between high-level streptomycin resistance and resistance to synergism seemed clear, the mechanism of this resistance was unknown.

Subsequent work showed that penicillin enhances the uptake of radiolabeled [^{14}C]streptomycin in strains with or without high-level streptomycin resistance (365). Therefore, it seemed unlikely that the resistance to synergism observed in strains with high-level streptomycin resistance was due to a permeability barrier. It was later demonstrated that an enterococcal strain that was highly resistant to streptomycin and resistant to penicillin/streptomycin synergism contained ribosomes that were insensitive to the in vitro effects of streptomycin on protein synthesis (i.e., streptomycin failed to inhibit the incorporation of radiolabeled phenylalanine into trichloroacetic acid-precipitable material and also failed to cause misreading) (608, 609). Thus, it seemed reasonable to postulate that the resistance to penicillin/aminoglycoside synergism observed in clinical isolates of enterococci might be due to the same mechanism. This hypothesis was consistent with the observation that all clinical enterococcal isolates examined at that time were susceptible to high levels of gentamicin (200 μg/mL) and to penicillin/gentamicin synergism (368).

However, the strain used in these ribosome studies was a laboratory mutant derived from a clinical isolate that had been susceptible to penicillin/streptomycin synergism and to high levels of streptomycin (MIC of 400 μg/mL) (609). Continued study of enterococci (*E. faecalis*) showed that 40 to 50% of clinical isolates in Boston had high-level resistance to both streptomycin and kanamycin and were likewise resistant to both penicillin/streptomycin and penicillin/kanamycin synergism (75). None of 203 isolates examined was found to harbor high-level resistance (MIC of more than 2000 μg/mL) to gentamicin, sisomicin, or tobramycin, and all 10 strains tested by the killing-curve technique were synergistically killed by penicillin plus gentamicin, sisomicin, or tobramycin (75). The findings with amikacin were at variance with those obtained with other aminoglycosides; although only one of 10 strains tested for synergism had high-level amikacin resistance (amikacin MIC of more than 2000 μg/mL), six of those 10 strains were resistant to synergistic killing by the penicillin/amikacin combination (the same six strains also exhibited high-level resistance to kanamycin [MIC of more than 2000 μg/mL]). Resistance to penicillin/amikacin synergism in enterococci was simultaneously reported by another group (36).

Additional study of clinical isolates with high-level streptomycin and kanamycin resistance revealed an explanation for these findings. The strains with high-level streptomycin and kanamycin resistance contained a 45-MDa plasmid that was transferable by conjugation and produced high-level resistance to both streptomycin and kanamycin and resistance to penicillin/streptomycin and penicillin/kanamycin synergism in a recipient strain that was previously susceptible (286). (The plasmid was identified by cesium chloride/ethidium bromide ultracentrifugation, by agarose gel electrophoresis, and by electron microscopy.) Curing with novobiocin produced simultaneous loss of the plasmid, of high-level aminoglycoside resistance, and of resistance to penicillin/aminoglycoside synergism (286). Thus, it seemed clear that—at least in some clinical isolates of enterococci—a conjugative plasmid was responsible for the observed resistance to penicillin/aminoglycoside synergism.

The mechanism of this plasmid-mediated resistance is not an altered ribosome. In vitro studies revealed no difference in the aminoglycoside susceptibility of ribosomes from the susceptible recipient strain and the resistant donor strain (a clinical isolate with high-level resistance) when studied with varying concentrations of streptomcyin, kanamycin, and amikacin (0.1 to 100 μg/mL) (285). Subsequently, aminoglycoside-inactivating enzymes, which explain the observed resistance to synergism, were found. A phosphotransferase in resistant strains inactivates kanamcyin and amikacin by phosphorylating the 3′-hydroxyl group, and an adenylyltransferase inactivates streptomycin (285). The absence of a 3′-hydroxyl group in gentamicin, tobramycin, netilmicin, and sisomicin protects them from phosphorylation by this enzyme. The adenylyltransferase that inactivates streptomycin has no activity against 2-deoxystreptamine-containing aminoglycosides, such as kanamycin, amikacin, gentamicin, tobramycin, netilmicin, and sisomicin.

Studies of the relative activities of these modifying enzymes with various aminoglycoside substrates also explain the apparent discrepancy between the infrequent occurrence of high-level amikacin resistance and the frequency of resistance to penicillin/amikacin synergism. Amikacin has a 3′-hydroxyl group that is phosphorylated by the *E. faecalis* phosphotransferase. However, this enzyme is less active against amikacin than kanamycin (285). This finding presumably explains why MICs of amikacin were generally lower than those observed with kanamycin (36, 75). Nevertheless, the activity of this enzyme is sufficient to confer resistance to penicillin/amikacin (as well as penicillin/kanamycin) synergism in strains with high-level kanamycin resistance.

Among strains of *E. faecalis* producing the 3′-phosphotransferase enzyme, combinations of penicillin or ampicillin with amikacin frequently are less bactericidal (slight to moderate degrees of antagonism by time-kill curves) than the β-lactam alone (528). Although a complete explanation of this phenomenon has not yet been elucidated, the answer in part appears related to the fact that against such isolates even concentrations of amikacin well above the MIC exert primarily a bacteriostatic effect, thus antagonizing the lethal effect of penicillin, which is greatest among growing cells (528).

The presence of a 3′-phosphotransferase in strains of enterococci with high-level resistance to streptomycin and kanamycin has also been reported in the absence of streptomycin-modifying enzymes (499). A minority of strains with high-level resistance to streptomycin do not have enzymes that inactivate streptomycin (by acetylation, phosphorylation, or adenylylation). The mechanism of their resistance has been elucidated and involves an altered ribosome (142), as noted in the laboratory mutant of *E. faecalis* studied earlier (609).

E. faecium strains are usually resistant to penicillin/tobramycin synergism (369), even though most *E. faecalis* strains that have been examined are susceptible to penicillin/tobramycin. Elaboration of low levels of a 6′-acetyltransferase enzyme (which inactivates tobramycin as well as sisomicin and netilmicin) by *E. faecium* explains resistance to penicillin/tobramycin synergism (105, 578). In contrast to the other aminoglycoside-inactivating enzymes described in enterococci, this activity is not transferable by conjugation and thus appears to be chromosomal (578). As noted above for *E. faecalis*, plasmid-mediated resistance to streptomycin and kanamycin has also been described in *E. faecium* (295). A more recently described 4′,4″-nucleotidyltransferase con-

fers high-level resistance to kanamycin and tobramycin, with resistance to synergism by amikacin combinations as well (78).

High-level resistance to gentamicin was unknown among enterococci prior to 1979, when it was first described in *E. faecalis* isolated in France (235). High-level resistance to gentamicin, with failure of penicillin/gentamicin synergism, can be attributed principally to the elaboration of a bifunctional enzyme with 6′-acetyltransferase and 2″-phosphotransferase activity to which the aminoglycoside is susceptible. Such isolates typically produce multiple aminoglycoside-modifying enzymes with adenylylating, acetylating, and phosphorylating activities (102). These enzymes are plasmid- or transposon-mediated and transmissible by conjugation to recipient strains in vitro (107, 230, 352). A single isolate of *E. faecalis* was described that resisted penicillin/gentamicin but not penicillin/tobramycin synergism (364). The mechanism involved in resistance to synergism appeared to be a specific defect in the transport of gentamicin (but not tobramycin). High-level gentamicin-resistant *E. faecalis* strains have now been isolated from centers around the world, and they comprise a significant fraction of clinical enterococcal isolates at some institutions (231). Characterization of the first reported clinical isolates of high-level gentamicin-resistant *E. faecium* has revealed enzymatically mediated resistance mechanisms completely analogous to those described among *E. faecalis* (146). A few enterococcal isolates that have moderate levels of gentamicin resistance (MICs of 256 to 1000 μg/mL) and are resistant to penicillin/gentamicin synergism have been encountered (27). These strains produce bifunctional enzyme but at levels that may be insufficient to yield MICs of more than 2000 μg/mL, at least in some media.

Resistance to the β-lactam component of β-lactam/aminoglycoside combinations has also been studied. Although combinations of cephalosporins and aminoglycosides are synergistic against enterococci in vitro (148, 165, 570, 572), experience with clinical use of cephalosporin/aminoglycoside combinations has generally been unsatisfactory (443). It has been suggested that the high MICs of cephalothin (16 to 32 μg/mL) and other cephalosporins against most enterococci may be a major factor in their lack of efficacy, because β-lactams typically do not produce synergism with aminoglycosides against enterococci unless they are present in concentrations near or greater than their MICs (538, 572).

Other studies have suggested that combinations of semisynthetic penicillinase-resistant penicillins (nafcillin, oxacillin, or methicillin) plus gentamicin are significantly less effective in vivo than penicillin/gentamicin, although their activities are similar to that of penicillin/gentamicin in vitro (311). The reason for this discrepancy is almost certainly related to the high levels of protein binding (80 to 92%) of nafcillin and oxacillin, because these drugs are much less effective (even with gentamicin) against enterococci when serum is added to the culture media in vitro (187).

In 1983, Murray and Mederski-Samoraj (374) described β-lactamase production in a clinical isolate of *E. faecalis*. Genetic determinants mediating production of this enzyme, which appears related to staphylococcal penicillinase by DNA hybridization techniques (375), were found to be transferable to recipient enterococcal strains by conjugation in vitro (374). Since that time, additional β-lactamase-producing isolates have been recovered in Philadelphia, New Haven (CT), Boston, and elsewhere (372, 378, 421, 452). Such isolates demonstrate a marked resistance to penicillin or ampicillin at high inocula. All but one of the isolates described to date have also been highly resistant to gentamicin. Elaboration of β-lactamase has been described in an *E. faecium* isolate (106). Resistance to penicillins (and other β-lactams) which is characteristic of the majority of clinical isolates of this species is usually due, however, to the presence of a low-affinity PBP (PBP-5) (582).

There is evidence of increasing resistance to β-lactams among *E. faecium* since the late 1980s. The concentration of penicillin required to inhibit 90% of isolates from one Boston hospital rose from 64 μg/mL in the 20-year period up to 1988 to 512 μg/mL for strains collected in 1989 and 1990 (200). Strains with very high levels of resistance to penicillin may fail to exhibit bactericidal synergism between penicillin and an aminoglycoside at clinically achievable concentrations of each (538).

Recently, strains of both *E. faecium* (297, 398, 581) and *E. faecalis* (493) (as well as a few isolates of other species) with resistance to vancomycin have been recovered from clinical specimens in Europe and the United States (256, 468). Resistance is frequently inducible upon incubation with vancomycin; induction of resistance is associated with the appearance of a new cell-membrane protein (approximately 39 to 39.5 kDa). At least in some isolates, this resistance trait is plasmid-mediated (297) and transferable to recipient strains by conjugation (297, 493).

Multiple genes involved in resistance to vancomycin have been localized to a large transposon (17). The net effect of activation of these genes is production of altered peptidoglycan precursors terminating in D-alanine-D-lactate (in *E. faecalis* and *E. faecium*), to which vancomycin binds with much less affinity than it does to its normal target, D-alanine-D-alanine.

The present model of resistance to synergism in enterococci (Fig. 9.24) relies primarily on the association between high-level resistance to an aminoglycoside and resistance to synergism with penicillin plus that aminoglycoside. The mechanism of high-level aminoglycoside resistance in clinical isolates of *E. faecalis* studied to date is usually synthesis of aminoglycoside-inactivating enzymes, but ribosomal resistance and other mechanisms play a role in some *E. faecalis* strains, especially those with high-level resistance to streptomycin alone. Mech-

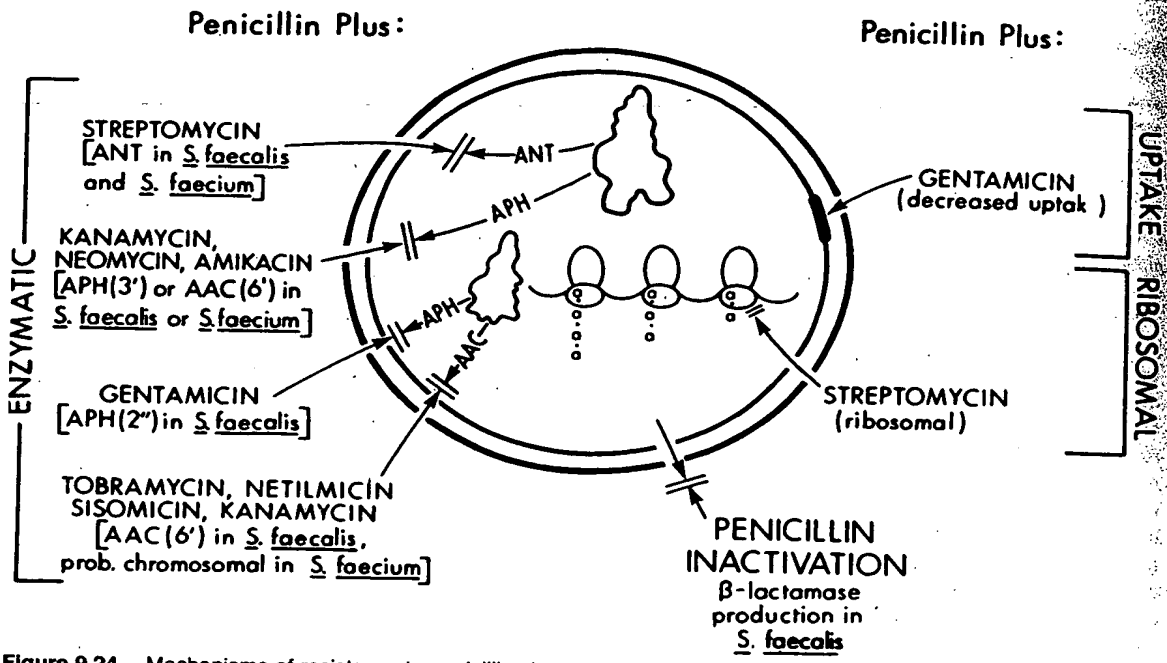


Figure 9.24. Mechanisms of resistance to penicillin plus aminoglycoside synergism against enterococci. Of these resistance mechanisms, the most prevalent is enzymatic inactivation. Ribosomal resistance to streptomycin also occurs. Enzymatic resistance is also plasmid-mediated, although the 6'-acetyltransferase in *E. faecium* is chromosomal. *ANT*, adenylyltransferase; *APH(3')*, 3'-phosphotransferase; *APH(2")*, 2"-phosphotransferase; *AAC(6')*, 6'-acetyltransferase. Not shown is the 4',4"-nucleotidyltransferase that confers resistance to synergism with kanamycin, tobramycin, and amikacin (78).

anisms of resistance to synergism in vivo (to combinations that are synergistic in vitro) are probably multiple. At present, the most important factors appear to be protein binding and the relationship of the MIC of the β-lactam or glycopeptide to achievable serum and tissue levels in vivo. However, the recent demonstration of transferable β-lactamase raises the ominous possibility that this trait could spread widely among the enterococci and obviate the utility of penicillin/aminoglycoside synergism.

Viridans Streptococci. Although these organisms are typically susceptible to penicillin/streptomycin synergism (132, 590), recent studies have revealed both high-level streptomycin resistance (154) and penicillin resistance (126a, 155) among clinical isolates. Both ribosomal (154) and enzyme-mediated (156) resistance mechanisms have been described that produce resistance to penicillin/streptomycin synergism. The penicillin resistance observed is associated with alterations in PBPs (155), similar to the alterations observed in the penicillin-resistant pneumococci initially isolated from the same areas of South Africa (213, 606). High-level gentamicin resistance has now also been detected in viridans group streptococci (260).

Other Genera. Resistance to penicillin/aminoglycoside synergism in other bacteria is presumably due to similar mechanisms, although studies to clarify the relative importance of aminoglycoside-resistant ribosomes, aminoglycoside-inactivating enzymes, permeability barriers, altered PBPs, and β-lactamases have rarely been performed. The limited data that are available suggest that, as the MIC of one of the drugs rises, the combination tends to become less effective. For example, with increasing resistance (MIC) to gentamicin, fewer strains of Enterobacteriaceae are susceptible to ampicillin/gentamicin and cephalothin/gentamicin synergism by the checkerboard technique (270). Similarly, the addition of carbenicillin or ticarcillin to gentamicin is ineffective against *P. aeruginosa* highly resistant to gentamicin (MIC of ≥80 μg/mL) (557). Likewise, a major factor in resistance to carbenicillin/aminoglycoside synergism in *A. calcoaceticus* is the aminoglycoside MIC; strains resistant to β-lactam/aminoglycoside synergism had MICs of ≥31 μg/mL for tobramycin (two of two strains) or ≥62.5 μg/mL for gentamicin (two of four strains) or kanamycin (two of two strains) (188). The mechanism of aminoglycoside resistance in these strains is the production of aminoglycoside-modifying enzymes (376, 377).

However, resistance to either the β-lactam or the aminoglycoside component of a given regimen does not completely preclude the possibility of a synergistic interaction. For example, in one study imipenem/amikacin synergism (MIC) was observed against 45% of imipenem-resistant *P. aeruginosa* (70), while another study noted piperacillin/amikacin synergism against 55% of

amikacin-susceptible and 88% of amikacin-resistant strains of this species (290).

Animal and Human Studies of Penicillin/ Aminoglycoside Synergism

Enterococci Use of the penicillin plus streptomycin combination for the treatment of enterococcal endocarditis began with the observation that this combination cured patients who had failed to respond to penicillin alone (239, 246). The effectiveness of penicillin plus streptomycin combinations has subsequently been documented in the rabbit endocarditis model (82, 284). Since the discovery of enterococcal strains with high-level streptomycin and kanamycin resistance (MICs of more than 2000 μg/mL) that are resistant to penicillin/streptomycin and penicillin/kanamycin synergism in vitro (366, 513), penicillin/gentamicin has been used widely for the treatment of patients with enterococcal endocarditis. Although there have been no controlled clinical trials, penicillin/gentamicin has been clearly shown to be effective for the treatment of enterococcal endocarditis due to strains with high-level streptomycin (and/or kanamycin) resistance in patients (571) and in the rabbit model (284). In addition, using the rabbit model, penicillin/streptomycin is no more effective than penicillin alone (and is significantly less effective than penicillin/gentamicin or penicillin/sisomicin) in the treatment of enterococcal endocarditis due to strains with high-level streptomycin resistance (82). The emergence of enterococci demonstrating high-level resistance to gentamicin (and other deoxystreptamine aminoglycosides) has posed a major therapeutic challenge. Although a minority of such isolates may prove to be not highly streptomycin-resistant (383), thus presumably permitting penicillin/streptomycin synergism, there are no known combinations of cell wall-active agents with clinically available aminoglycosides that would be synergistic for the majority of such organisms. Some patients with documented or presumed endocarditis due to highly gentamicin-resistant organisms have responded to a cell wall-active agent alone or as an adjunct to surgery (166, 313, 361, 420).

As discussed above, there is no role for currently available cephalosporins or antistaphylococcal penicillins, alone or in combination with any aminoglycoside, in the treatment of serious enterococcal infections. There is no evidence that imipenem (20) or any of the acylaminopenicillins (145) is superior to penicillin or ampicillin in combination regimens for the treatment of enterococcal infections. Imipenem, vancomycin, or ampicillin plus sulbactam may play a role in the treatment of infections due to β-lactamase-producing strains (224), but such regimens are limited by the fact that most β-lactamase-producing strains have also been highly gentamicin resistant, as noted earlier.

Other Streptococci. Animal studies have suggested that penicillin/aminoglycoside combinations might also be useful in the treatment of nonenterococcal streptococcal infections. Penicillin plus streptomycin was more effective than either drug alone against viridans group streptococcal endocarditis in the rabbit model (i.e., it produced more rapid sterilization of vegetations) (470), and ampicillin plus gentamicin produced increased survival, compared with either drug alone, in a group B streptococcal mouse peritonitis model (124). Penicillin plus streptomycin has also been shown to be more effective than penicillin alone in the prevention of viridans group streptococcal endocarditis in the rabbit model (133). In another animal model of endocarditis due to strains of *Streptococcus sanguis*, superiority of the penicillin/streptomycin combination to penicillin alone was demonstrable only against penicillin-tolerant or relatively resistant (penicillin MIC of 1 μg/mL) strains; no advantage was noted against a fully penicillin-susceptible strain against which the single β-lactam was highly effective (584).

In spite of these studies, the role of penicillin/aminoglycoside combinations in the treatment of viridans streptococcal endocarditis in humans remains controversial. Relapse rates of 6 to 15% were initially reported after 2 weeks of treatment with penicillin alone (216). However, it has not been convincingly demonstrated that penicillin/streptomcyin therapy for viridans streptococcal endocarditis (216, 535, 537, 590) is more effective than treatment for 4 weeks with penicillin alone (234, 259). Nonetheless, combination therapy may allow a shorter duration of treatment (i.e., 2 weeks) for patients with uncomplicated endocarditis due to fully penicillin-susceptible organisms (583).

Staphylococci. Animal studies utilizing penicillin-susceptible strains have shown penicillin/gentamicin to be more effective than penicillin alone in the treatment of *S. aureus* peritoneal infection in mice (515) and endocarditis in rabbits (471). Nafcillin/gentamicin has been found to be more effective than nafcillin alone in the treatment of endocarditis due to penicillinase-producing *S. aureus* in the rabbit model (469). Retrospective studies and a large, controlled, clinical trial of nafcillin/gentamicin versus nafcillin alone in *S. aureus* endocarditis have failed to provide evidence for superior efficacy of nafcillin/gentamicin combinations in the therapy of human *S. aureus* endocarditis (1, 283), but combination therapy may allow shorter treatment courses in injection drug uses with *S. aureus* right-sided endocarditis (126).

Pseudomonas. Animal studies have shown that combinations of carbenicillin or ticarcillin plus gentamicin or tobramycin are more effective than the individual drugs alone in the treatment of *Pseudomonas* peritonitis in normal rats (11, 12), in the treatment of *Pseudomonas* peritonitis and bacteremia in neutropenic rats (489), and in the therapy of *Pseudomonas* peritonitis in mice (101). Several clinical studies on the use of antibiotic combinations for the therapy of Gram-negative infections, especially in neutropenic or immunosuppressed patients, have demonstrated that the results of treatment with synergistic combinations (defined by reduction of

the MIC to one fourth or less of its original value for each of the two drugs in the combination, i.e., a FIC index of ≤0.5) are superior to those in which synergism did not occur (269, 273, 294). Each of these studies included infections due to *P. aeruginosa* that responded well to synergistic therapy, but none contained enough patients with *Pseudomonas* infections alone to determine whether synergistic combinations significantly increased the survival of patients with *Pseudomonas* infection. In the study by Anderson et al. (8), 10 of 12 patients with *P. aeruginosa* bacteremia who received synergistic antimicrobial therapy responded, whereas none of six responded to nonsynergistic regimens. However, in a retrospective analysis of more than 400 cases of *P. aeruginosa* bacteremia (in both neutropenic and nonneutropenic patients), no differences in response were noted among patients treated with an appropriate antipseudomonas β-lactam alone, compared to the β-lactam plus an aminoglycoside (49). These and other studies stress the importance of treatment with a β-lactam active against the pathogen, at the very least (139). If high serum bactericidal titers can be achieved with such an agent alone (which may not always be the case), the added benefit of synergistic therapy becomes less clear. Reyes et al. (451) studied, in vitro, strains of *P. aeruginosa* isolated from patients with endocarditis, and they concluded that synergism between drugs used for treatment (carbenicillin plus gentamicin or tobramycin) was necessary for, but not a guarantee of, cure with medical therapy.

In a prospective study of 200 patients with *P. aeruginosa* bacteremia, the mortality rate was significantly lower in patients receiving combination therapy (antipseudomonal β-lactam plus aminoglycoside in all but one case), compared with those receiving monotherapy (27% versus 47%) (223). Whether the combination used was synergistic against the infecting organism (FIC of ≤0.5 or FIC of ≤1.0 or by killing curves at one fourth the MIC of each) did not significantly influence outcome, although there was a trend toward improved survival in combinations demonstrating bactericidal synergism. Notably, there was incomplete correlation between results of in vitro studies using different methods. In another study, addition of rifampin to antipseudomonal β-lactam/aminoglycoside combinations resulted in increased bacteriologic eradication rates, but not in survival, of patients with *P. aeruginosa* bacteremia (282).

Other Gram-Negative Bacteria. Animal studies have shown that ticarcillin and carbenicillin enhance the activity of gentamicin or tobramycin against *E. coli* or *E. cloacae* in the mouse peritonitis model (101). Confirmatory evidence from human studies is restricted to the studies of Gram-negative septicemia cited above, which involve many Enterobacteriaceae, including *E. coli*, *Citrobacter*, *Klebsiella*, *Enterobacter*, and *Proteus*, as well as *P. aeruginosa* (269, 273, 294). Several studies have also supported the value of combination therapy for Gram-negative rod bacteremia in neutropenic patients. One study noted a 75% response among patients receiving two drugs active against the pathogen but only a 44% response rate for organisms susceptible to only one drug (277). A large multicenter trial comparing two-drug regimens of amikacin plus ticarcillin, azlocillin, or cefotaxime noted a 66% response when organisms were susceptible to both components but only a 21% response when the strain was resistant to the β-lactam (271). DeJongh et al. (122) observed that synergism (by MIC checkerboard) was important for patients with persistent profound (less than 100/μL) neutropenia.

On the other hand, other trials have found little benefit in adding an aminoglycoside to cefoperazone (435) or aztreonam (253) for the treatment of Gram-negative rod bacteremia (including patients who were neutropenic). The apparent success of such single-drug regimens employing the newer β-lactams may relate to the high serum bactericidal titers against many pathogens that can be attained using these agents (551).

Nevertheless, because of high mortality rates associated with Gram-negative sepsis—particularly in neutropenic patients—and because of the necessity to start empirical antibiotic therapy early (often before susceptibility results are known, and certainly before synergy studies can be done or serum bactericidal titers established), many clinicians still prefer to use β-lactam/aminoglycoside combinations in this setting. A multicenter trial comparing ceftazidime plus amikacin administered for 3 days with ceftazidime plus amikacin administered for 9 days for the treatment of Gram-negative bacteremia in neutropenic cancer patients found improved response with the longer course of combination therapy (81% versus 48%) (151). In a prospective observational study of *Klebsiella* spp. bacteremia in 230 patients, outcome was similar for patients receiving monotherapy with an active agent and for those receiving combination therapy (almost always β-lactam plus aminoglycoside) (281). However, mortality rates were significantly lower for combination-treated patients with hypotension (24% versus 50%). Antibiotic interactions in vitro were not reported.

Although the possible role of synergistic therapy in treatment of *A. calcoaceticus* var. *antitratus* infection has been raised (189), there have been no controlled animal or human trials to study the question in vivo.

Combinations of Antimicrobial Agents That Act by Other or Unknown Mechanisms

This section considers combinations of antimicrobials that synergize against bacteria by unknown mechanisms. Therefore, the discussion of some of these is restricted to available laboratory and clinical studies, because both the mechanisms of synergistic activity and the mechanisms of resistance to synergism are unknown.

Laboratory Studies of Antimicrobial Combinations That Act by Other or Unknown Mechanisms

Combinations of Agents That Target the Bacterial Ribosome. RP 59500 is a combination of two semisynthetic pristinamycin derivatives, RP 57669 or quinupristin (derived from pristinamycin I_A) and RP 54476 or dalfopristin (derived from pristinamycin II_A) (21). Combining the two agents results both in inhibitory synergism against various Gram-positive organisms (54, 392) and in bactericidal activity lacking with the individual compounds. It has been proposed that binding of the II_A component to the ribosome induces a conformational change that increases binding of the I_A component, resulting in a very stable ternary complex that blocks protein synthesis (21).

Clindamycin plus Gentamicin. Utilizing the checkerboard technique, clindamycin/gentamicin has been shown to be synergistic against 33 of 62 Enterobacteriaceae and *P. aeruginosa* strains that were susceptible to ≤6.2 μg/mL gentamicin (158). However, the concentrations of clindamycin required for synergism were frequently 50 to 100 μg/mL, which is significantly greater than clinically achievable levels (5 to 25 μg/mL) (163). Other studies also suggested that the clindamycin plus gentamicin combination was synergistic against *E. coli* and certain other Enterobacteriaceae when tested by a modified checkerboard technique (303). However, the use of techniques that measure the early bactericidal activity of amikacin and gentamicin has demonstrated antagonism between clindamycin and the aminoglycoside (610). Our own data support the latter observations. We have noted decreased early killing of a number of Gram-negative bacilli, including *S. marcescens*, with the combination of clindamycin plus gentamicin versus gentamicin alone. With gentamicin alone there was often overgrowth of gentamicin-resistant colonies after 24 hours of incubation. With clindamycin plus gentamicin such colonies did not emerge, and at 24 hours the colony counts in tubes containing clindamycin plus gentamicin were often lower than those in tubes that contained gentamicin alone (RC Moellering Jr and CBG Wennersten, unpublished observations). Thus, the net effect of the combination (at 24 hours) is increased killing (presumably due to the decreased emergence of resistant mutants), despite early antagonism (in the first few hours). This effect may account for the "synergism" noted by investigators who used an overnight incubation for the checkerboard determination (303).

Clindamycin/gentamicin has been shown to be effective in the treatment of polymicrobial peritoneal infection in a rat model, preventing both early death from septicemia and late abscess formation (320). In addition, this combination has been shown to be effective in treating mixed aerobic-anaerobic infections in humans (96). However, in this situation, each drug is presumably acting against a different organism, i.e., it probably does not represent an example of synergism as defined at the start of this chapter.

Clindamycin/gentamicin synergism has also been reported against some strains of viridans streptococci (132). However, this combination has produced antagonism against other streptococci (502), and the clinical significance of these observations remains to be established. The clinical data cited above suggest that early antagonism noted in time-kill curve determinations may not be important with normal patients, although studies with immunosuppressed patients (the group for whom antagonism might be most important) have not been reported. In vitro synergism, of uncertain mechanism and clinical significance, has been reported between clindamycin and gentamicin against *Chlamydia trachomatis* (423).

Polymyxins plus Sulfonamides or TMP. Polymyxins are effective in vitro against most aerobic and facultative Gram-negative bacilli, except *Proteus* and *Serratia* spp. (202). However, when combined with a sulfonamide or TMP, which are also ineffective alone, polymyxins synergistically inhibit both *Proteus* and *Serratia* spp. Synergism of colistin (polymyxin E) and a sulfonamide against *Proteus* spp. was first noted in 1959 (222) and was later confirmed by several other investigators (410, 543). Most *S. marcescens* strains have also been shown to be susceptible to polymyxin B/sulfonamide synergism. Strains that were resistant to polymyxin B/sulfonamide synergism have been killed by polymyxin B plus TMP (202). TMP, SMZ, and polymyxin B have been studied alone and in combination against a number of Gram-negative bacilli (458). Successful in vitro combinations included SMZ plus polymyxin B against *Klebsiella-Enterobacter-Serratia* spp. (21 of 26 strains demonstrated either synergism or an additive effect), TMP plus polymyxin B against *Proteus-Providencia* spp. (14 of 14 strains), and all three drugs together against nine of 12 strains studied by the killing-curve technique (458).

Although the mechanism of this interaction has not been established, the ability of TMP to synergize with polymyxins against strains resistant to sulfonamide/polymyxin synergism suggests that the folate pathway is involved. In addition, two lines of evidence imply that the function of the sulfonamide may be to allow polymyxin to reach the cell membrane. (*a*) There is a lag of four generation times for the synergistic bactericidal effect of the combination after the addition of sulfonamide. The same lag occurs if both drugs are added together or if the sulfonamide is added first, followed by colistin four generations later, in which case the effect occurs immediately after the addition of colistin (409). (*b*) Artificial lipid membranes prepared from *Proteus* spp. resistant to polymyxins in vitro are as susceptible to disruption by polymyxin B in the laboratory as are those prepared from sensitive strains (202).

Thus, the available information suggests that sulfonamides and/or TMP may act on the cell through the folate pathway to increase the access of polymyxin to the cell membrane. The mechanism by which these effects occur remains unknown. Polymyxin combinations with sulfonamides and/or TMP have received very little clinical attention because of polymyxin toxicity, and they are unlikely to be studied in the near future.

TMP plus Aminoglycosides. Preliminary studies have shown that TMP plus amikacin may produce synergism against Enterobacteriaceae that are susceptible to TMP (MIC of ≤1.6 μg/mL) and amikacin (MIC of ≤6.4 μg/mL) alone (415). Although most strains studied were examined by the checkerboard technique, killing curves also showed that some strains were synergistically killed by this combination.

Metronidazole plus Clindamycin. Metronidazole and clindamycin synergize at clinically achievable concentrations (0.125 to 1.0 and 2.0 μg/mL, respectively) against a majority of *B. fragilis* strains tested (65).

Quinolones and Other Antimicrobials. Synergism between ciprofloxacin and aminoglycosides (214) and various β-lactams (215) is infrequently demonstrable against Enterobacteriaceae, and antagonism seems quite rare. Against *P. aeruginosa*, combinations of ciprofloxacin with aminoglycosides demonstrate synergism against less than one third of isolates (94, 114, 183), whereas combination with various β-lactams (e.g., azlocillin, cefpiramide, or imipenem) results in synergism more frequently (10 to 70% for the β-lactams listed) (114, 172, 183, 459). Ciprofloxacin plus ceftazidime or aztreonam also yielded synergistic activity against *P. aeruginosa*, especially when the organism was resistant to the fluoroquinolone (71). Combinations of enoxacin, ofloxacin, or norfloxacin with coumermycin (a drug that inhibits the B-subunit of DNA gyrase) were found to be synergistic against most strains of *S. aureus* examined (393). However, in a rat *S. aureus* endocarditis model ciprofloxacin/coumermycin was less effective than was the fluoroquinolone alone (428). Novobiocin, another agent that acts at the level of DNA gyrase, inhibited the bactericidal activities of ciprofloxacin and ofloxacin against *S. aureus* and *Staphylococcus warneri* in vitro (308). Addition of rifampin to ciprofloxacin tends to decrease the bactericidal activity of the latter against *S. aureus*, but the combination does appear to suppress the emergence of rifampin-resistant colonies (212, 549). Against vancomycin-resistant enterococci, combinations of ciprofloxacin with vancomycin sometimes yield inhibitory synergism, but at concentrations of the former too high to be of likely importance (547). Studies to date suggest that such interactions may be related to diminished induction of proteins mediating resistance (547). Combinations of the investigational fluoroquinolones sparfloxacin or clinafloxacin with either streptomycin or gentamicin resulted in bactericidal synergism against some enterococcal isolates studied by time-kill methods (427). High concentrations of ampicillin combined with ciprofloxacin have been shown to produce a bactericidal effect against some strains of enterococci resistant to both classes of agents (291). Mechanisms of such interactions have not been determined.

Antineoplastic Agents plus Known Antimicrobials. Several investigators have shown that antineoplastic agents such as bleomycin, mitomycin C, and 5-fluorouracil, some of which demonstrate antibacterial activity at attainable serum concentrations (47), can synergize with known antimicrobials (such as aminoglycosides and β-lactams) against *S. aureus* (244) and Gram-negative bacilli (546, 595). Although some of these interactions are bactericidal, their clinical significance is incompletely defined.

SUMMARY

Mechanisms of Antimicrobial Interactions Resulting in Synergism (Table 9.7)

There are presently four well-established mechanisms of antimicrobial interaction that produce synergism:

1. Sequential inhibition of a common biochemical pathway (e.g., TMP plus SMZ, amdinocillin plus a β-lactam, and vancomycin plus a β-lactam).
2. Inhibition of β-lactamase or decreased production of β-lactamase (e.g., clavulanic acid or sulbactam plus penicillin or chloramphenicol plus penicillin).
3. Sequential inhibition of cell wall synthesis (a variation of the sequential inhibition of a common biochemical pathway) (e.g., amdinocillin or vancomycin plus a β-lactam).
4. Use of β-lactams or other agents acting on the cell wall to permit increased entry of aminoglycosides (e.g., penicillin, carbenicillin, vancomycin, or imipenem plus streptomycin or gentamicin).

Other mechanisms are theoretically possible, such as a conformational change of a ribosomal binding site induced by one drug that enhances the binding of another, as has been suggested for quinupristin/dalfopristin (21). Possible directions for future research include not only the use of different β-lactams, β-lactamase inhibitors, aminoglycosides, and folate antagonists to overcome the known mechanisms of resistance to antimicrobial synergism but also the use of new combinations of agents that have different mechanisms of action.

Mechanisms Resulting in Antimicrobial Antagonism

Four mechanisms of antimicrobial interaction are known to produce antimicrobial antagonism:

1. Combination of bacteriostatic agents with β-lactams (e.g., penicillin plus tetracycline or chloramphenicol).
2. Combination of 50S subunit ribosomal inhibitors

Table 9.7
Examples of Synergistic Antimicrobial Combinations—In Vitro[a]

Organism	Mechanism of Synergism	Antimicrobial Combination(s)	Technique and Modifications	Effect Measured	Reference(s)
		Gram-Negative Bacilli			
Acinetobacter calcoaceticus (*Herellea vaginicola*)	β-Lactam + aminoglycoside	Carbenicillin + kanamycin, tobramycin, or gentamicin	Killing curve	Bactericidal	(188)
		Imipenem + amikacin	Checkerboard-broth microtiter	Bacteriostatic	(196)
	Unknown	Ciprofloxacin + azlocillin	Checkerboard-broth microtiter	Bacteriostatic	(371)
Citrobacter	Sequential enzyme inhibition	TMP + SMZ	Checkerboard-agar dilution (fractional inhibitory concentration (FIC) index)	Bacteriostatic	(68)
	Inhibition of protective enzymes	Clavulanic acid or sulbactam + ampicillin or amoxicillin	Checkerboard-broth microtiter	Bacteriostatic	(150, 175)
		Chloramphenicol + ampicillin, cephaloridine, or carbenicillin	Cellophane transfer	Bacteriostatic	(355)
	Inhibition of cell wall synthesis	Amdinocillin + ampicillin, cefoxitin, or cefamandole	Checkerboard-agar dilution	Bacteriostatic	(390)
	β-Lactam + aminoglycoside	Amikacin + apalcillin, azlocillin, ceftazidime, or ceftriaxone	Checkerboard-broth microtiter	Bacteriostatic	(196)
Enterobacter	Sequential enzyme inhibition	TMP + SMZ	Checkerboard-agar dilution (FIC index)	Bacteriostatic	(68)
	Inhibition of protective enzymes	Nafcillin + penicillin, ampicillin, or cephalothin	Checkerboard-broth dilution	Bacteriostatic	(158)
		Clavulanic acid or sulbactam + ampicillin	Checkerboard-broth microtiter and agar dilution	Bacteriostatic	(174, 446)
		Chloramphenicol + ampicillin, cephaloridine, or carbenicillin	Cellophane transfer	Bacteriostatic	(355)
		Chloramphenicol + cephaloridine	Killing curve	Bactericidal	(355)
		Clindamycin + cefamandole	Killing curve	Bactericidal	(479)
	Inhibition of cell wall synthesis	Adminocillin + amoxicillin	Checkerboard-broth and agar dilution	Bacteriostatic	(389)
		Adminocillin + ceftazidime	Checkerboard-broth microtiter with sampling	Bacteriostatic and bactericidal	(550)
	β-Lactam and aminoglycosidase	Ampicillin or cephalothin + gentamicin	Checkerboard-agar dilution	Bacteriostatic	(270)
		Amikacin + piperacillin, cephalothin, moxalactam, cefotaxime, or cefoxitin	Killing curve	Bactericidal	(191, 193)
		Amikacin + imipenem, aztreonam, or cefmenoxime	Checkerboard-broth microtiter	Bacteriostatic	(196)
Enterobacteriaceae (misc.)	Inhibition of protective enzymes	Cefoperazone + sulbactam	Checkerboard-broth microtiter	Bacteriostatic	(251)

Table 9.7—*continued*

Organism	Mechanism of Synergism	Antimicrobial Combination(s)	Technique and Modifications	Effect Measured	Reference(s)
		Gram-Negative Bacilli—*continued*			
Enterobacteriaceae (misc.)—*continued*		Ampicillin + sulbactam, clavulanate, or tazobactam	Broth microdilution	Bacteriostatic	(431)
	β-Lactam + aminoglycoside	Moxalactam, cefoperazone, cefotaxime, or ceftazidime + amikacin	Checkerboard-broth microtiter	Bacteriostatic	(252)
Escherichia coli	Sequential enzyme inhibition	TMP + SMZ	Checkerboard-broth and agar dilution, paper strip diffusion	Bacteriostatic	(2)
	Inhibition of protective enzymes	Cloxacillin or dicloxacillin, + ampicillin, penicillin, mezlocillin, or cephalothin	Checkerboard-broth agar dilution	Bacteriostatic	(131, 402, 466, 525)
		Clavulanic acid or sulbactam + ampicillin, amoxicillin, piperacillin, or cephaloridine	Checkerboard-broth microtiter and agar dilution	Bacteriostatic	(150, 174, 349, 396, 446, 588)
		Clavulanic acid or sulbactam + adminocillin (adminocillin-resistant isolates)	Checkerboard-agar dilution	Bacteriostatic	(391)
		Sulbactam + cefoperazone	Checkerboard-broth dilution	Bacteriostatic	(251)
		Chloramphenicol + ampicillin, cephaloridine, or carbenicillin	Cellophane transfer	Bacteriostatic	(355)
	Inhibition of cell wall synthesis	β-Chloro-D-alanine + penicillin	Killing curve	Bactericidal	(504)
		Adminocillin + amoxicillin or ampicillin	Checkerboard-broth and agar dilution	Bacteriostatic	(389, 545)
			Killing curve	Bactericidal	(389)
		Fosmidomycin + cefazolin, cephalexin, ampicillin, or ticarcillin	Checkerboard-agar dilution	Bacteriostatic	(397)
	β-Lactam + aminoglycoside	Ampicillin or cephalothin + gentamicin	Checkerboard-agar dilution	Bacteriostatic	(270, 345)
		Piperacillin, cefotaxime, or cefamandole + amikacin or gentamicin	Killing curve	Bactericidal	(191, 193)
	Unknown	Clindamycin + gentamicin	Checkerboard-broth dilution	Bacteriostatic	(303)
Klebsiella	Sequential enzyme inhibition	TMP + SMZ	Checkerboard-agar dilution	Bacteriostatic	(68)
	Inhibition of protective enzymes	Cloxacillin + ampicillin or cephalothin	Checkerboard-broth and agar dilution	Bacteriostatic	(131, 466)
		Clavulanic acid or sulbactam + ampicillin, amoxicillin, cefotaxime, ceftriaxone, aztreonam, or piperacillin	Checkerboard-broth microtiter and agar dilution	Bacteriostatic	(150, 174, 175, 396, 587, 603)
		Chloramphenicol + ampicillin, cephaloridine, or carbenicillin	Cellophane transfer	Bacteriostatic	(353)
		Clavulanic acid + amdinocillin (against amdinocillin-resistant isolates)	Checkerboard agar dilution	Bacteriostatic	(391)

		amdinocillin-resistant isolates)			
	Inhibition of cell wall synthesis	Fosmidomycin + cefazolin, cephalexin, or ticarcillin	Checkerboard dilution agar	Bacteriostatic	(397)
		Amdinocillin + cephalothin, cefazolin, amoxicillin, ampicillin, or ceftazidime	Checkerboard-broth and agar	Bacteriostatic	(25, 389, 550)
			Killing curve	Bactericidal	(390)
		Vancomycin + cephalothin or carbenicillin	Checkerboard-broth microtiter	Bacteriostatic	(127)
	β-Lactam + aminoglycoside	Cephalothin + gentamicin or amikacin	Checkerboard-agar dilution and broth microtiter	Bacteriostatic	(270, 345)
		Cefazolin + amikacin	Checkerboard-agar dilution and broth microtiter with sampling	Bacteriostatic and bactericidal	(274)
		Apalcillin, cefmenoxime, or ceftazidime + amikacin	Checkerboard-broth microtiter	Bacteriostatic	(196)
		Piperacillin, cefotaxime, cefamanadole, cefoxitin, cephalothin, or moxalactam + amikacin	Killing curve	Bactericidal	(191, 193)
Morganella morganii	β-Lactam + aminoglycoside	Imipenem + amikacin	Checkerboard-agar dilution	Bacteriostatic	(149)
Proteus spp.	Inhibition of protective enzymes	Nafcillin, oxacillin, or methicillin + ampicillin	Checkerboard-broth dilution	Bacteriostatic	(466)
		Clavulanic acid or sulbactam + ampicillin, amoxicillin, cephalodrine, carbenicillin, or ticarcillin	Checkerboard-broth microtiter and agar dilution	Bacteriostatic	(131, 150, 175, 176, 446, 587)
		Chloramphenicol + ampicillin, cephaloridine, or carbenicillin	Cellophane transfer	Bacteriostatic	(355)
		Acetohydroxaminic acid + methenamine (in urine)	Killing curve	Bactericidal	(382)
	Inhibition of cell wall synthesis β-Lactam and aminoglycoside	Amdinocillin + ampicillin, cephalothin, cephradine, cefazolin, or carbenicillin	Checkerboard-broth and agar dilution	Bacteriostatic	(25, 263, 383)
		Cephalothin, carbenicillin, or ampicillin + gentamicin	Checkerboard-agar dilution	Bacteriostatic	(270, 272)
		Azlocillin, aztreonam, ceftazidime, or ceftriaxone + amikacin	Checkerboard-broth microtiter	Bacteriostatic	(196)
		Piperacillin, cefotaxime, or moxalactam + amikacin	Killing curve	Bactericidal	(191, 193)
Providencia spp.	Inhibition of protective enzymes	Chloramphenicol + ampicillin, cephaloridine, or carbenicillin	Cellophane transfer	Bacteriostatic	(355)
		Sulbactam + ampicillin	Checkerboard-broth microtiter	Bacteriostatic	(150)
Pseudomonas aeruginosa	Inhibition of protective enzymes	Methicillin, cloxacillin, or nafcillin + penicillin or ampicillin	Checkerboard-broth dilution	Bacteriostatic	(466)
		Clavulanic acid or sulbactam + azlocillin or ampicillin	Checkerboard-broth dilution	Bacteriostatic	(76, 150)
	Inhibition of cell wall synthesis	Vancomycin + cephalothin or carbenicillin	Checkerboard-broth microtiter	Bacteriostatic	(127)

Table 9.7—*continued*

Organism	Mechanism of Synergism	Antimicrobial Combination(s)	Technique and Modifications	Effect Measured	Reference(s)
		Gram-Negative Bacilli—*continued*			
Pseudomonas aeruginosa—*continued*	β-Lactam + aminoglycosides	Carbenicillin, ticarcillin, piperacillin, moxalactam, ceftriaxone, ceftazidime, cefotaxime, azlocillin, cefsulodin, imipenem, ceftazidime, cefpiramide, mezlocillin, cefpimizole, cefipime, or aztreonam + gentamicin, tobramycin, amikacin, netilmicin, or sisomicin	Checkerboard-agar dilution, broth microtiter, and broth dilution with sampling	Bacteriostatic	(10, 16, 53, 70, 89, 101, 143, 160, 178, 184, 196, 221, 261, 276, 278, 290, 324, 340, 344, 358, 379, 401, 490, 520, 599, 611)
			Killing curve	Bactericidal	(59, 61, 89, 101, 272, 395, 401, 430, 433, 503, 513, 520, 561, 600)
	Unknown	Clindamycin + gentamicin	Checkerboard-broth microtiter	Bacteriostatic	(162)
		Ciprofloxacin + imipenem, azlocillin, cefoperazone, aztreonam, ceftazidime, or vancomycin	Checkerboard-broth dilution	Bacteriostatic	(70, 71, 114, 118, 371, 459)
		Ciprofloxacin + imipenem, or amikacin	Killing curve	Bactericidal	(183)
Pseudomonas cepacia	β-Lactam + aminoglycoside	Piperacillin or aztreonam + amikacin	Checkerboard-agar dilution	Bacteriostatic	(16)
		Aztreonam + gentamicin or tobramycin	Checkerboard-broth microtiter with sampling	Bacteriostatic Bactericidal	(53) (53)
Xanthomonas maltophilia	Unknown	Rifampin + gentamicin + carbenicillin	Checkerboard-broth microtiter	Bacteriostatic	(601)
		Rifampin + TMP + SMZ + carbenicillin	Checkerboard-broth microtiter	Bacteriostatic	(601)
		Ciprofloxacin + mezlocillin, piperacillin, imipenem, aztreonam, ceftazidime, cefoperazone, or gentamicin	Checkerboard-agar dilution	Bacteriostatic	(97)
	β-Lactam + aminoglycoside	Ceftazidime + tobramycin	Checkerboard-broth microtiter	Bacteriostatic	(89)
Salmonella typhi	Sequential enzyme inhibition	TMP + SMZ	Checkerboard-agar dilution	Bacteriostatic	(72)
Salmonella typhimurium	Inhibition of protective enzymes	Chloramphenicol + ampicillin, cephaloridine, or carbenicillin	Cellophane transfer	Bacteriostatic	(355)
	Inhibition of cell wall synthesis	β-Chloro-D-alanine + penicillin	Killing curve	Bactericidal	(504)
		Amdinocillin + ampicillin	Killing curve	Bactericidal	(390)
			Checkerboard-broth dilution	Bacteriostatic	(74)
Salmonella spp.	Inhibition of protective enzymes	Sulbactam + ampicillin	Checkerboard-broth microtiter	Bacteriostatic	(150)

Serratia marcescens	Inhibition of protective enzymes	Sulbactam + ampicillin	Checkerboard-broth microtiter	Bacteriostatic	(150)
		Clavulanic acid or sulbactam + amdinocillin (versus amdinocillin-resistant isolates)	Checkerboard-agar dilution	Bacteriostatic	(391)
		Chloramphenicol + ampicillin, cephaloridine, or carbenicillin	Cellophane transfer	Bacteriostatic	(355)
	Inhibition of cell wall synthesis	Fosmidomycin + cefazolin, cephalexin, ampicillin, or ticarcillin	Checkerboard-agar dilution	Bacteriostatic	(397)
		Amdinocillin + cephradine	Checkerboard-broth dilution	Bacteriostatic	(263)
		Amdinocillin + ceftazidime	Checkerboard-broth microtiter	Bacteriostatic	(550)
			dilution with sampling	Bactericidal	(550)
		Vancomycin + cephalothin or carbenicillin	Checkerboard-broth microtiter	Bacteriostatic	(127)
	β-Lactam + aminoglycoside	Piperacillin, moxalactam, cefotaxime, or cefoperazone + amikacin	Checkerboard-agar dilution	Bacteriostatic	(290)
		Azlocillin, aztreonam, ceftazidime, ceftriaxone, cefmenoxime, imipenem, or moxalactam + amikacin or netilmicin	Checkerboard-broth microtiter	Bacteriostatic	(196, 339)
	Unknown	Sulfadiazine + polymyxin B	Checkerboard-broth dilution	Bacteriostatic	(202)
Shigella	Sequential enzyme inhibition	TMP + SMZ	Checkerboard-broth and agar dilution	Bacteriostatic	(460)
	Inhibition of protective enzymes	Sulbactam + ampicillin	Checkerboard-agar dilution	Bacteriostatic	(174)
		Chloramphenicol + ampicillin, cephaloridine, or carbenicillin	Cellophane transfer	Bacteriostatic	(355)
	Inhibition of cell wall synthesis	Amdinocillin + ampicillin	Killing curve	Bactericidal	(390)
		Gram-Negative Coccobacilli			
Haemophilus influenzae	Sequential enzyme inhibition	TMP + SMZ	Checkerboard-agar dilution	Bacteriostatic	(68)
	Inhibition of cell wall synthesis	Amdinocillin + ampicillin	Checkerboard-agar dilution	Bacteriostatic	(588)
	Inhibition of protective enzymes	Nafcillin + ampicillin	Checkerboard-broth dilution	Bacteriostatic	(597)
		Clavulanic acid or sulbactam + ampicillin, amoxicillin, or ticarcillin	Checkerboard-agar dilution, broth microtiter, and broth microtiter with sampling	Bacteriostatic	(150, 159, 176, 437, 450, 588, 597)
				Bactericidal	(159, 450, 598)
	Unknown	Rifampin + TMP	Checkerboard-broth microdilution	Bactericidal	(328)
		Roxithromycin + sulfamethoxazole	Checkerboard-broth microtiter with sampling	Bacteriostatic and bactericidal	(293)
Branhamella catarrhalis	Inhibition of protective enzymes	Clavulanic acid + amoxicillin	Checkerboard-agar dilution	Bacteriostatic	(238)
	Sequential enzyme inhibition	TMP + SMZ	Checkerboard-agar dilution	Bacteriostatic	(456)

Table 9.7—*continued*

Organism	Mechanism of Synergism	Antimicrobial Combination(s)	Technique and Modifications	Effect Measured	Reference(s)
		Gram-Negative Coccobacilli—*continued*			
Neisseria gonorrhoeae	Sequential enzyme inhibition	TMP + SMZ (1:1)	Checkerboard-agar dilution	Bacteriostatic	(448)
	Inhibition of protective enzymes	Clavulanic acid or sulbactam + ampicillin, amoxicillin, or ticarcillin	Checkerboard-broth microtiter	Bacteriostatic	(150, 176, 238, 437)
Bordetella pertussis	Sequential enzyme inhibition	TMP + SMZ	Checkerboard-agar dilution	Bacteriostatic	(73)
		Gram-Negative Anaerobes			
Bacteroides fragilis group	Inhibition of protective enzymes	Cefoxitin + carbenicillin, piperacillin, or mezlocillin	Checkerboard-agar dilution	Bacteriostatic	(26)
		Clavulanic acid or sulbactam + cefoperazone, cefsulodin, ampicillin, amoxicilin, ticarcillin, piperacillin, or penicillin G	Checkerboard-agar dilution and microtiter broth dilution with sampling	Bacteriostatic	(109, 150, 159, 173, 176, 177, 238, 251, 396, 450, 587)
				Bactericidal	(159, 173, 450)
		Tazobactam + ceftriaxone or piperacillin	Agar dilution	Bacteriostatic	(592)
	Unknown	Metronidazole + clindamycin	Checkerboard-agar dilution, and broth dilution microtiter with sampling	Bacteriostatic and bactericidal	(65)
		Ciprofloxacin + clindamycin, mezlocillin, cefoxitin, or cefotaxime	Checkerboard-broth microtiter or agar	Bacteriostatic	(153, 580)
Bacteriodes melaninogenicus	β-Lactam + aminoglycoside	Penicillin + gentamicin	Checkerboard-agar dilution	Bacteriostatic	(56)
	Inhibition of protective enzymes	Clavulanic acid + ticarcillin	Checkerboard-broth microdilution	Bacteriostatic	(177)
		Tazobactam + ceftriaxone or piperacillin	Agar dilution	Bacteriostatic	(592)
		Gram-Positive Cocci			
Viridans streptococci	β-Lactam + aminoglycoside	Penicillin + streptomycin	Checkerboard-broth dilution with sample	Bactericidal	(132)
			Killing curve	Bactericidal	(590)
Diphtheroids	Cell wall-active agent + aminoglycoside	Vancomycin or teicoplanin + gentamicin or tobramycin	Killing curve	Bactericidal	(373, 506)
		Daptomycin + gentamicin or tobramycin	Killing curve	Bactericidal	(505)

Group A streptococci	β-Lactam + aminoglycoside	Penicillin + gentamicin	Checkerboard-broth microdilution with sampling at 3 and 6 hr	Bactericidal	(24)
			Killing curve		(24)
Group B streptococci	β-Lactam + aminoglycoside	Ampicillin or penicillin + gentamicin	Checkerboard-broth microdilution with sampling at 3 and 6 hr	Bactericidal	(24, 483, 484)
			Killing curve	Bactericidal	(24, 483, 484)
Pneumococci	Inhibition of cell wall synthesis	Cefotaxime + fosfomycin	Killing curve	Bactericidal	(28)
Enterococci	β-Lactam (or other agent acting on the cell wall) + aminoglycoside	Penicillin, ampicillin, carbenicillin, nafcillin, oxacillin, or vancomycin + streptomycin, kanamycin, gentamicin, tobramycin, amikacin, netilmicin, or sisomicin	Killing curve, and checkerboard-broth dilution with sampling	Bactericidal	(75, 164, 190, 242, 284, 310, 338, 367–369, 563)
		Imipenem + gentamicin, tobramycin, or amikacin	Killing curve	Bactericidal	(144, 196, 566)
	Inhibition of cell wall synthesis	Daptomycin + imipenem or fosfomycin	Checkerboard-broth microdilution	Bacteriostatic	(119)
			Killing curve	Bactericidal	(119)
		Imipenem + fosfomycin or teicoplanin	Checkerboard-broth microdilution	Bacteriostatic	(120)
			Killing curve	Bactericidal	(120)
	Inhibition of cell wall synthesis ($Vanco^R$)	Glycopeptides + various β-lactams	Checkerboard-microdilution	Bacteriostatic	(84)
			Checkerboard-microdilution Double-disk	Bacteriostatic	(296)
		Amoxicillin + cefotaxime	Checkerboard-agar dilution	Bacteriostatic	(333)
	Unknown	Imipenem + rifampin	Checkerboard-microdilution	Bacteriostatic	(120)
			Killing curve	Bactericidal	(120)
	Unknown ($Vanco^R$)	Ciprofloxacin + vancomycin	Checkerboard-microdilution	Bacteriostatic	(547)
		Ciprofloxacin + ampicillin	Killing curve	Bactericidal	(291)
		Sparfloxacin or clinafloxacin + gentamicin	Killing curve	Bactericidal	(427)
Staphylococcus aureus	Inhibition of protective enzymes	Clavulanic acid or sulbactam + ticarcillin, piperacillin, ampicillin, amoxicillin, or penicillin G	Checkerboard-broth microtiter and agar dilution	Bacteriostatic	(131, 150, 238, 396, 446, 450, 588)
		Chloramphenicol + penicillin G	Cellophane transfer	Bacteriostatic	(355)
	Inhibition of cell wall synthesis	Daptomycin + aztreonam or ceftriaxone	Checkerboard-broth microdilution	Bacteriostatic	(494)

Table 9.7—*continued*

Organism	Mechanism of Synergism	Antimicrobial Combination(s)	Technique and Modifications	Effect Measured	Reference(s)
		Imipenem + teicoplanin or fosfomycin	Checkerboard-broth microdilution	Bacteriostatic	(120)
			Killing curve	Bactericidal	(120)
		Imipenem + vancomycin or teicoplanin	Checkerboard-broth microdilution with sampling	Bacteriostatic Bactericidal	(342)
	β-Lactam (cell-wall agent) + aminoglycoside	Nafcillin, oxacillin, cephalothin, or vancomycin + kanamycin, gentamicin, tobramycin, netilmicin, or sisomicin	Killing curve	Bactericidal	(62, 268, 562, 564, 565)
		Daptomycin + tobramycin	Checkerboard-broth microdilutoin	Bacteriostatic	(494)
	Unknown	Teicoplanin + rifampin	Checkerboard-broth microtiter	Bacteriostatic and bactericidal	(540)
		Clindamycin or imipenem + rifampin	Killing curve	Bactericidal	(120, 228)
		Ciprofloxacin + azlocillin	Checkerboard-broth microdilution	Bacteriostatic	(371)
		Levofloxacin + oxacillin	Killing curve	Bactericidal	(416)
S. aureus (penicillin-susceptible)	β-Lactam + amino-glycoside	Penicillin + gentamicin	Checkerboard-broth dilution	Bacteriostatic	(515)
S. aureus (methicillin-resistant)	Inhibition of protective enzymes	Sulbactam + ampicillin or penicillin G	Checkerboard-broth dilution	Bacteriostatic	(450)
	Unknown	Teicoplanin + rifampin	Checkerboard-broth dilution	Bacteriostatic and bactericidal	(540)
		Fusidic acid + rifampin	Killing curve	Bactericidal	(157)
	Inhibition of cell wall synthesis	Fosfomycin + methicillin or cefamandole	Checkerboard-broth microdilution	Bacteriostatic	(6)
		Imipenem + cefotiam, cefpiramide, piperacillin	Checkerboard-agar Killing curve	Bacteriostatic Bactericidal	(346)
		Vancomycin + cefoperazone or cefpirome	Checkerboard-agar	Bacteriostatic	(491)
		Meropenem + various β-lactams	Checkerboard-agar	Bacteriostatic	(542)
		Cefotiam + cefmetazole, imipenem, vancomycin and others	Checkerboard-agar	Bacteriostatic	(523)
Coagulase-negative staphylococci	Inhibition of protective enzymes	Sulbactam + ampicillin	Checkerbroad-broth microtiter	Bacteriostatic	(450)
	β-Lactam (or other agent acting on the cell wall) + aminoglycoside	Vancomycin + ampicillin or tobramycin	Killing curve	Bactericidal	(321, 565)

		Daptomycin + tobramycin	Checkerboard-broth microdilution	Bacteriostatic	(494)
	Inhibition of cell wall synthesis	Daptomycin + aztreonam or ceftriaxone	Checkerboard-broth microdilution	Bacteriostatic	(494)
		Imipenem + fosfomycin or teicoplanin	Checkerboard-broth microdilution	Bacteriostatic	(120)
			Killing curve	Bactericidal	(120)
		Imipenem + vancomycin or teicoplanin	Checkerboard-broth microdilution with sampling	Bacteriostatic Bactericidal	(342)
	Unknown	Vancomycin + rifampin	Killing curve	Bactericidal	(321)
		Gentamicin + rifampin	Killing curve	Bactericidal	(321)
		Imipenem + rifampin	Checkerboard-broth microtiter	Bacteriostatic	(120)
			Killing curve	Bactericidal	(120)
Coagulase-negative-staphylococci (methicillin-resistant)	Inhibition of protective enzymes	Sulbactam + ampicilln or penicillin G	Checkerboard-broth dilution	Bacteriostatic	(450)
		Gram-positive Bacilli			
Lactobacilli	β-Lactam + amino-glycoside	Penicillin or ampicillin + streptomycin, or gentamicin	Checkerboard-broth dilution with sampling	Bactericidal	(38)
Listeria monocytogenes	β-Lactam + amino-glycoside	Ampicillin or penicillin + streptomycin or gentamicin	Killing curve	Bactericidal	(326, 363)
		Imipenem + gentamicin	Killing curve	Bactericidal	(144)
	Unknown	Rifampin + penicillin G, ampicillin, or cefamandole	Checkerboard-broth microtiter with sampling	Bactericidal	(541)
		Rifampin + erythromycin	Checkerboard-broth microtiter	Bacteriostatic	(541)
		Trimethoprim + gentamicin	Killing curve	Bactericidal	(326)
		Actinomycetes			
Nocardia asteroides	Sequential enzyme inhibition	TMP + SMZ	Checkerboard	Bacteriostatic	(332)
	Inhibition of protective enzymes	Clavulanic acid + penicillin, ampicillin, carbenicillin, mezlocillin, piperacillin, or amdinocillin	Checkerboard-agar dilution Paper strip	Bacteriostatic Bacteriostatic	(267) (267)
	Inhibition of cell wall synthesis	Imipenem + cefotaxime	Checkerboard-agar dilution	Bacteriostatic	(195)
	Unknown	TMP + SMZ + imipenem	Checkerboard-agar dilution	Bacteriostatic	(195)
		TMP + SMZ + amikacin	Checkerboard-agar dilution	Bacteriostatic	(195)

Table 9.7—*continued*

Organism	Mechanism of Synergism	Antimicrobial Combination(s)	Technique and Modifications	Effect Measured	Reference(s)
		Mycobacteria			
Mycobacterium avium complex	Unknown	Ethambutol + rifampin, streptomycin, kanamycin, amikacin, clarithromycin or ciprofloxacin	Checkerboard-agar dilution	Bacteriostatic	(31, 262, 607)
		Rifampin + streptomycin, kanamycin, isonazid, or clarithromycin			
		Isonazid + streptomycin or kanamycin			
		Streptomycin + ethambutol, rifampin + ethambutol, or INH, rifampin + ethambutol	Growth rate in broth	Bacteriostatic	(232)
		Ethambutol + sparfloxacin ± rifampin	Broth dilution	Bacteriostatic Bactericidal	(593)
Mycobacterium fortuitum	Inhibition of protective enzymes	Clavulanic acid + amoxicillin or cephalothin	Checkerboard-agar dilution	Bacteriostatic	(110)
	Unknown	Ciprofloxacin + amikacin, or TMP + SMZ	Checkerboard-agar dilution	Bacteriostatic	(182)
Mycobacterium tuberculosis	Inhibition of protective enzymes	Clavulanic acid + amoxicillin	Broth dilution with sampling	Bactericidal	(111)
	Unknown	Teicoplanin + ethambutol	Checkerboard-broth dilution	Bacteriostatic	(15)
Mycobacterium kansasii	Unknown	Ethambutol + ciprofloxacin, rifampin, or isoniazid	Growth rate in broth	Bacteriostatic	(227)
Mycobacterium leprae	Unknown	Rifabutin + clinafloxacin or sparfloxacin	Checkerboard measurement of metabolic activity	Bacteriostatic Bactericidal	(125)
		Fungi			
Paracoccidioides brasiliensis	Inhibition of protective enzymes	TMP + SMZ (1:5)	Checkerboard-broth dilution	Fungistatic	(516)
Aspergillus	Unknown	Amphotericin B + rifampin	Checkerboard-broth dilution	Fungistatic	(123)

[a]Demonstration of synergism is often strain and concentration dependent. Other effects, including antagonism, can result from several of the combinations described as showing synergism.

(e.g., erythromycin plus clindamycin) (in vitro data only).

3. Combination of aminoglycosides with bacteriostatic agents (e.g., gentamicin plus tetracycline or chloramphenicol).
4. Combination of β-lactams containing one agent that derepresses β-lactamase production (e.g., cefoxitin plus another β-lactam).

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